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Essential Fatty Acid Metabolism and Lipid Mediator Generation and Binding in Cultured Human Airway Epithelial Cells

By

Jing Xuan Kang ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of **Doctor of Philosophy.**

In

Medical Sciences (Medicine)

Edmonton, Alberta
Spring 1994

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(SIGNED) Kang Jingxuan

Permanent Address:
#208, 12215 - Lansdowne Drive
Edmonton, Alberta
Canada T6H 4L4

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Essential Fatty Acid Metabolism and Lipid Mediator Generation and Binding in Cultured Human Airway Epithelial Cells** submitted by **Jing Xuan Kang** in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** in **Medical Sciences (Medicine).**

M.T. Clandinin (Supervisor)

S.F.P. Man

N.E. Brown

D.N. Brindley

C.J. Field

V.C. Dias

A.B.R. Thomson

B.J. Holub (External examiner)

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ABSTRACT

Cultured human airway epithelial cells were assayed to examine 1) the capacity to incorporate and desaturate exogenous essential fatty acid, 18:2ω6; 2) turnover of the phospholipid fatty acyl chains; 3) effect of blocking Cl^- conductance on 18:2ω6 metabolism; 4) regulation of platelet-activating factor (PAF) binding to the cells by exogenous fatty acids and ion channel blockers; 5) modulation of PAF production by modification of cellular fatty acid composition.

Cultured human airway epithelial cells readily took up exogenous fatty acids from the culture medium and incorporated them into cellular phospholipid and triglyceride. 18:2ω6 was rapidly desaturated and elongated to form 20:4ω6. Fatty acyl chains of the phospholipids were in a dynamic state of rapid turnover with a half life of about 5-7 h (PC > PI >PE > PS). The fatty acid composition of epithelial membrane could be readily modified by exogenous fatty acid supplementation.

Blocking the chloride-channel with 9-AC inhibited essential fatty acid incorporation into phospholipid. Inhibition of Cl^- conductance across cell membrane appeared to alter fatty acid incorporation and was specific to 18:2ω6. Lipid analysis revealed that 9-AC could induce a fatty acid profile similar to that found in cystic fibrosis.

A single class of specific PAF binding sites with a K_d of 1.8 ± 0.2 nM and Bmax of 21.0 ± 2.1 fmol/10^6 cells was identified. PAF binding to the cell induced a rise in the intracellular free calcium concentration. Supplementation of the cells with 20:5ω3 inhibited specific PAF binding and PAF-induced intracellular calcium mobilization, while supplementation of the cells with 16:0 increased PAF binding to the membrane. Calcium and sodium channel blockers inhibited PAF binding while blocking the chloride channel increased PAF binding to the cell.

Human lung epithelial cells (A549) were found to synthesize PAF upon stimulation with calcium ionophore A23187. Production of PAF was enhanced by enrichment of the membrane phospholipid with 20:4ω6, but was decreased by

supplementation of the medium with 20:5ω3. A number of 20:4ω6 metabolites specifically $PGF_{2\alpha}$, PGE_2, LTB_4, LTD_4 and LTE_4 were found to stimulate PAF generation.

In conclusion, this thesis demonstrates that 1) human airway epithelial cells actively incorporate and metabolize exogenous fatty acids; 2) Cl^- conductance may play a role in fatty acid incorporation into membrane phospholipid of the epithelial cell; 3) the epithelial cell has functional receptors for PAF and PAF binding activity can be altered by exogenous fatty acids and ion channel blockers; 4) synthesis of PAF by the cell can be modulated by changing membrane fatty acid composition. These results provide insight into the role of airway epithelial cells and exogenous fatty acids in physiological and pathological processes of pulmonary tissues.

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Chapter I

INTRODUCTION

Modification of membrane fatty acid composition has an important impact on normal cell function and disease (Clandinin et al, 1991). Since the degree of fatty acid metabolism is an important determinant of membrane fatty acid composition and differs between animal species, organs and cell types, characterization of fatty acid metabolism in a particular cell type is important to understanding of regulation of cell membrane fatty acid profile. Definition of a specific factor affecting fatty acid metabolism in a particular cell type could provide insight into abnormality of fatty acid content of membrane in certain disease states such as cystic fibrosis. This thesis is designed 1) to characterize essential fatty acid metabolism in human airway epithelial cells and to examine the effect of inhibition of Cl^- conductance on fatty acid metabolism in these cells; 2) to determine the effect of modification of cellular fatty acid composition on synthesis and binding activity of platelet-activating factor in the epithelial cell.

Membrane Lipids and Cell Function

Current understanding of the organization of biological membranes relies on the fluid mosaic model proposed by Singer and Nicholson (1972) in which proteins are embedded to varying degrees in a lipid bilayer. Motion of functional proteins within the membrane may be required for specific functions. This concept, together with observations that membrane proteins are lipid-dependent has indicated a tight or close association of membrane lipid properties with protein functions (reviewed by Kimelberg, 1977; Stubbs and Smith, 1984). Thus, membrane proteins which serve as enzymes, receptors, carriers or ion-channels might be expected to show changes in activity in response to variation in the lipid composition in the microenvironment

of the protein (Clandinin et al., 1985, 1991; Stekhoven and Bonting, 1981).

Fatty acid modification of membrane lipid has formed the basis for experiments in which fatty acid composition, membrane physical properties and membrane function are simultaneously compared to determine the nature of relationships between these properties (reviewed by Whale 1983). Previous studies using cell culture and model membrane systems have elucidated relationships between these properties and membrane function in tissues. In this regard, the relationship of polyunsaturated fatty acids and membrane fluidity and function has been thoroughly reviewed (Stubbs and Smith, 1984; Brenner, 1984; Clandinin et al, 1985; Spector, 1985; Yeagle, 1989). Utilizing animal models, it has been illustrated that diet-induced alterations in membrane fatty acid composition are associated with demonstrable changes in the function of specific membrane proteins (Clandinin et al., 1985, 1991; McMurchie, 1988), particularly the binding activity of membrane receptors such as the insulin receptor (Field et al., 1990), thyroid hormone receptor (Venkatraman et al., 1986) and adenosine receptor (Kang et al., 1992).

The effect of membrane lipids on cell function lies not only in their role in the structure of membrane, but also in their independent effect as modulators of cell functions. Lipid modulators of cell function have been recently reviewed by Merrill (1989) and by Galli et al (1993). Membrane lipids, in addition to functioning as determinants of membrane structure and anchors for membrane-associated proteins, also function as ligands for cell-surface receptors and as "second messengers" of signal transduction. Cell stimulation by a number of agonists triggers cleavage of membrane lipid through activation of phospholipases, especially the A_2 and C, to yield: unsaturated fatty acids, particularly arachidonic acid, which are converted to eicosanoids; diacylglycerols, which activate protein kinase C; inositol phosphates, which stimulate release of calcium from intracellular stores; and lysoalkylphosphatidylcholine, which is converted to platelet-activating factor, a potent inflammatory mediator (Merrill, 1989; Galli et al, 1993). It has been shown that the

levels of ω6 and ω3 polyunsaturated fatty acid in cell phospholipid modulate the generation of lipid derived mediators after in vitro stimulation (Galli et al, 1993). However, precise relationships between individual fatty acids and generation and action of these lipid mediators remains to be examined.

In summary, membrane lipids play very important roles in cell function. Modification of membrane lipids has important implications for normal cell function and disease. Thus, the understanding of fatty acid metabolism of a particular cellular or subcellular fraction is of significance.

Factors Affecting Fatty Acid Composition of Cell Membrane

Fatty acid incorporation (deacylation-reacylation)

The fatty acid contained in cell lipids, particularly the membrane phospholipids, undergoes continuous tunrover (Spector et al., 1981a; Innis and Clandinin, 1981; Rosenthal, 1987b). If fatty acid is present in the extracellular fluid, it will exchange with the intracellular fatty acid that is turning over as a result of mixing in the free fatty acid pool. Fatty acids taken up by cells and activated to corresponding fatty acyl-coA esters are then incorporated into cellular phospholipids (Scheme I.1). This process can occur as part of the deacylation and reacylation of existing phospholipids or during de novo phospholipid synthesis. The relative contribution of these two routes varies greatly with the cell types and between individual fatty acids (Rosenthal, 1987a). To a great extent, the deacylation-reacylation reaction determines the characteristic pattern of fatty acid composition of phospholipids in different tissues (Lagarde et al., 1985; Kaya et al., 1984).

The reactions of deacylation and reacylation depend on activities of a number of acyltransferases, transacylases and phospholipases. Each of these enzymes has its own substrate specificity (Thompson and Martin, 1984). Phospholipases A_1 and A_2 cleave a fatty acid from the sn-1 and sn-2 positions of a phospholipid, respectively,

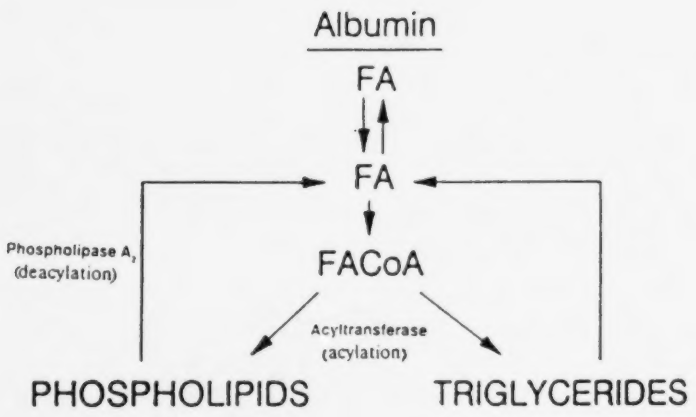

Fig.I.1 Turnover of intracellular fatty acids. Abbreviations used: FA, fatty acid; FACoA, fatty acyl coenzyme A. The fatty acid contained in cell lipids, particularly the membrane phospholipids, undergoes continuous turnover. If fatty acids are available in the extracellular fluid, they will mix the intracellular fatty acid undergoing turnover. Through this process, the intracellular fatty acid composition, including the fatty acid composition of the membrane phospholipids, can change somewhat to reflect type of fatty acid available in the diet.

allowing the potential for reacylation with a different profile of fatty acids (Holub and Kuksis, 1978; Van Den Bosch, 1980). Phospholipase A_2 has been found as a membrane-associated enzyme (van den Bosch, 1980). This phospholipase may be regulated by Ca^{++} ions and calmodulin (Wong and Cheung, 1979) and shows some specificity for the type of unsaturated fatty acid at the sn-2 position (Waite and Sisson, 1971). Following phospholipase action , deacylated phopholipids are available for reacylation by the action of fatty acyl transferases, utilizing as substrates fatty acyl-CoAs derived from dietary sources or de novo synthesis and acylating in a specific manner at either the sn-1 and sn-2 positions (Holub and Kuksis, 1978).

Compositional characteristics, such as fatty acyl chain length and degree of saturation and unsaturation, as well as the nature of the phospholipid head, determine the specificity of these acylating enzymes (Lands, 1979; Holub and Kuksis, 1978). These factors, along with the substrate pool of fatty acyl-CoAs available for reacylation, account for the positional specificity and unique fatty acid profile observed with the various classes of phospholipids present in mammalian membranes.

In short, the capacity of a particular cellular fraction to incorporate exogenous fatty acid into membrane phospholipid via above processes has important effects on membrane fatty acid composition. Although the control of enzymes responsible for fatty acid esterification is not well understood, it is conceivable that any factor causing reduction of incorporation of exogenous polyunsaturated fatty acid (e.g. 18:2ω6) into phospholipid may result in significant alteration in membrane fatty acid profile (ex. low levels of essential fatty acid).

Fatty acid desaturation and elongation

Desaturation and elongation activity plays an important role in determining fatty acid composition of phospholipid, because polyunsaturated fatty acids must be formed by elongation and desaturation of the essential fatty acids linoleic and linolenic acid, which are available in the diet (Figure I.2). Saturated and monounsaturated fatty acids can also be desaturated and elongated. Thus, the relative availability of saturated and unsaturated fatty acids for membrane phospholipid synthesis is determined by dietary fat composition and activity of desaturase enzymes.

Δ9-, Δ6-, and Δ5-desaturases catalyze dehydrogenation of fatty acids in corresponding positions of carbon chains (acyl-CoA). It is generally accepted that 18:2ω6 and 18:3ω6 are metabolized, respectively, to 22:5ω6 and 22:6ω3. The last step in these reaction sequences requires a Δ4-desaturase (Figure I.2). However, the presence of a Δ4-desaturase has never really been established. Recently, Voss and

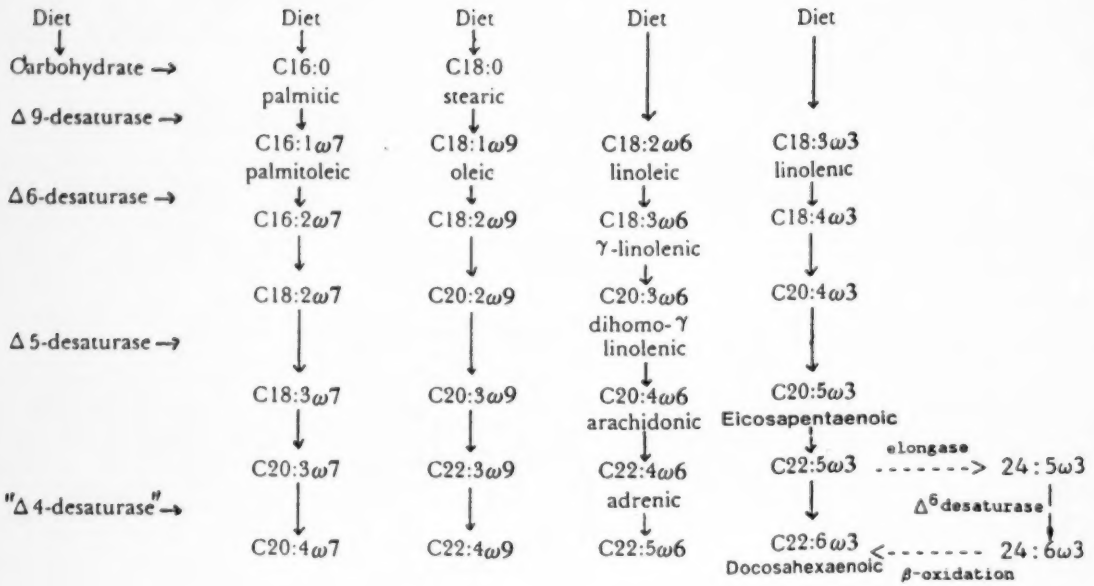

Figure I.2. Fatty acid desaturation and elongation

Sprecher et al. (1991) have demonstrated that the formation of 22:5ω6 and 22:6ω3 is independent of Δ4-desaturase, alternatively through a retroconversion pathway (Figure I.2). The Δ6 desaturase is considered to be a rate-limiting step in the biosynthetic pathway leading to the formation of 20:4ω6 (Holloway et al., 1963; Brenner, 1984). The enzyme is bound to the microsomal membrane, requires lipid for its activity (Brenner et al., 1966) and may be associated with other proteins bound to the membrane (Jeffcoat et al., 1984). Enzyme activity can be affected by many other factors. The concentration of cytoplasmic protein may modulate activity by organization of the enzyme complex in the membrane (Jomer et al., 1979). The effect of metal ions on desaturation has been reported (eg. copper and zinc can

increase desaturase activity; Jeffcoat et al., 1984). Recent studies show that dietary ω3 fatty acid supplementation impairs the desaturation of 18:2ω6 and decreases 20:4ω6 level in plasma and tissue (Garg, 1988a; Garg, 1988b). Metabolic hormones, eg. glucagon and epipinephrine via cAMP decrease, while insulin increases Δ6-desaturase activity (Brenner, 1981). Other physiological changes such as aging (Takahashi et al., 1991) and fasting (Brenner, 1981) have been associated with reduced Δ6-desaturase activity.

Desaturation and elongation of 18:2ω6 provides 20:4ω6 for synthesis of eicosanoids which are the group of physiologically and pharmacologically active compounds known as prostaglandins, thromboxane and leukotrienes (Bergstrom, 1981). Certainly physiological control mechanisms that alter desaturation and elongation and incorporation of essential fatty acid will alter eicosanoids metabolism.

Dietary fat

There is convincing evidence in animal models that change in diet fat content and/or composition could alter cell membrane fatty acid composition in various tissue types (reviewed by Clandinin et al., 1985, 1991; McMurchie, 1988). The fatty acid profile of the major membrane phospholipids has been shown to be readily and rapidly modified by dietary lipids, with significant changes occuring within 2-3 days in rat heart mitochondria (Innis and Clandinin, 1981). In intestinal mucosa, a relatively short period of feeding (7 days) is sufficient to change fatty acid composition of mucosal membrane lipid. When essential fatty acid intake was adequate, a low P/S (polyunsaturated/saturated) diet increased the 16:0 content of membrane lipids, whereas high P/S diets increased ω6 fatty acid level in mucosal membrane lipids (Clandinin et al., 1985; Garg et al., 1987). For liver plasma membrane, a dose-response relationship was shown to exist between the fatty acid content (18:2ω6) of the diet and fatty acid tail composition of membrane

phospholipid (Neelands and Clandinin, 1983). In adipose tissue, diet significantly altered the fatty acid composition of the major adipocyte plasma membrane phospholipids in both control and diabetic animals (Field and Clandinin, 1984). Feeding a high polyunsaturated fat diet increased the total polyunsaturated fatty acid content and P/S ratio in all membrane phospholipids analyzed from control animals. In diabetic animals the tendency to higher content of polyunsaturated fatty acid in phospholipid for animals fed the high P/S diet was also evident. In liver nuclear envelope, high dietary linoleic acid levels increased incorporation of 18:2ω6 and 20:4ω6 into liver nuclear envelope phospholipids compared with animals fed the low linoleic acid diet (Venkatraman et al., 1986; Kang et al., 1992). In brain, the nature of dietary fat fed was found to produce changes in brain microsomal and synaptic plasma membrane phospholipid fatty acid composition (Foot et al., 1982). Similarly, rats fed a diet rich in ω6 fatty acids produced a high ratio of ω6/ω3 fatty acids in synaptosomal membrane phospholipid (Hargreaves and Clandinin, 1987). Changes in diet fat content and/or composition alter cell membrane composition possibly via 1) alteration in the rates of de novo synthesis of phospholipids (Hargreaves and Clandinin, 1987a); 2) redistribution of fatty acyl chains via phospholipase and acyltransferase (Van den Bosch, 1980); 3) direct desaturation of membrane phospholipid-linked fatty acds (Garg et al., 1988).

In summary, the capacity of a particular cellular or subcellular fraction to incorporate exogenous fatty acid, desaturate/elongate available fatty acid substrate, degrade existing lipid and synthesize new lipid will determine in situ control of membrane phospholipid composition. Dietary fat is an important factor influencing lipid composition via the above processes.

Basic Defect of Cystic Fibrosis and Essential Fatty Acid Status in CF

Basic defect of cystic fibrosis

Cystic fibrosis (CF) is the most common petentially lethal autosomal recessive disorder of Caucasians, affecting 1 in 2500 newborns. It is characterized by abnormalities of water and electrolyte transport in the epithelial cell that lead to pancreatic and pulmonary insufficiency (Taussig, 1984). Recent progress in understanding of the genetic and functional basis of CF has provided a foundation for defining its molecular pathology as well as developing novel therapies (Collins, 1992).

Isolation of the gene for CF (Rommens et al., 1989; Riordan et al., 1989; Kerem et al., 1989) has provided insight into the molecular basis of the disease. The gene responsible for CF contains 250,000 base pairs of genomic DNA localized on chromosome 7 at q31. It encodes a protein of 1,480 amino acids called the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989). CFTR was predicted to contain five domains (Figure I.3): two membrane-spanning domains,

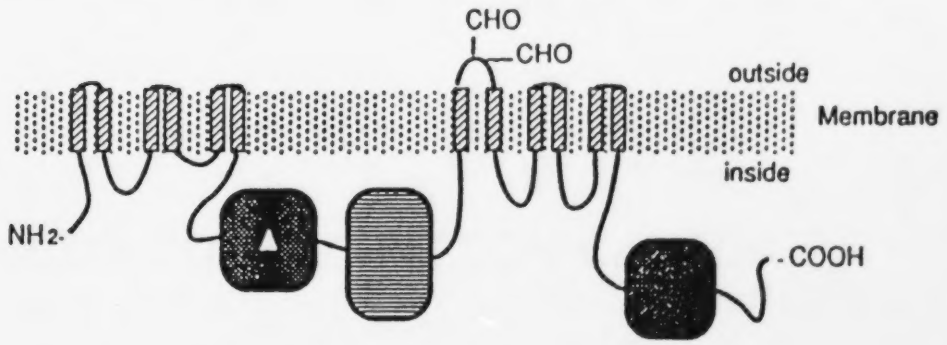

Fig. I.3 Model of CFTR and its localization in membrane. Each of the two membrane-spanning domains has three extrior loops. First loop in second membrane-spanning domain is predicted to have two N-linked glycosylation sites. Two nucleotide-binding domains and a regulatory domain are also present in CFTR. Greek letter Δ represents location of ΔF508 mutation. (Modified from Tsui LC, Buchwald M [Adv Hum Genet 1991;20:153-266].)

each composed of six transmembrane segments; an R domain, which contains several consensus phosphorylation sequences; and two nucleotide-binding domains, which were predicted to interact with ATP (Riordan et al, 1989). Sequence analysis of the CFTR gene of CF chromosomes has revealed a variety of mutations (about 200 mutations) (Cutting et al., 1990; White et al., 1990; Dean et al., 1990; Kerem et al., 1990). Extensive population studies have indicated that the most common CF mutation, a deletion of the 3 nucleotides that encode phenylalanine 508 (ΔF508) is present on 70% of all CF chromosomes, but never on normal chromosomes (Kerem et al., 1989). The remaining CF alleles appear diverse. At present about 200 different mutations have been reported. Approximately half of these are missense mutations, leading to substitutions of single amino acids in the protein. Another significant proportion are nonsense mutations, expected to cause premature termination of CFTR polypeptide and resulting in a truncated, nonfunctional protein. The different mutations result in three genotypes of CF (ΔF508/ΔF508; ΔF508/non-ΔF508; non-ΔF508/non-ΔF508). These genotypes are associated with variation of the clinical presentation, severity of disease and rate of progression of CF (Tizzano and Buchwald, 1992).

Functional expression of the CF defect reduces the chloride ion permeability of epithelial tissues (Quinton, 1990). The ability of epithelial cells in the airways, sweat glands, pancreas and other tissues to secrete Cl^- in response to cAMP-mediated agonists is lost or severely reduced. Activation of apical membrane Cl^- channels by cAMP-dependent protein kinase is impaired (Frizzell, 1987; Welsh, 1990). Many studies have suggested that the defect in the Cl^- channel may lie in a regulatory protein that transduces the effects of protein kinase activation (Frizzell, 1987; Welsh, 1990). Abnormalities in epithelial sodium transport in CF cells (Boucher et al., 1986) support the concept of a regulatory defect that can also affect other cellular functions.

However, the most recent work reported by Anderson et al. (1991) and by

Kartner et al. (1991), showing that expression of the CF gene in nonepithelial cells endows them with a plasma membrane Cl^- conductance that can be switched on by cAMP, implies that the CFTR which is defective in CF is a chloride channel. This conclusion is supported by other evidences suggesting that transfer of the normal CFTR cDNA to CF epithelial cells can correct the CF defect (Drumm et al, 1990) and that incorporation of purified CFTR into planar lipid bilayers produces a Cl^- channel (Bear et al, 1992). Recent studies also indicate that CFTR may carry out other important functions such as intracellular acidification (Barasch et al, 1991) and cAMP-mediated endocytosis and exocytosis (Bradbury et al, 1992).

This basic defect provides insight into the pathogenesis of CF pulmonary diseases. Hyposecretion of Cl^- (perhaps plus increase in Na^+ absorption) in the airway epithelium due to the CFTR mutation results in dehydration of mucus. This impairs airway clearance and leads to increase in susceptibility to infection by *Pseudomonas*. Consequently, inflammatory response to the chronic infection causes airway injury and other severe complications (Collins, 1992). However, the relationship of the basic defect to other abnormalities such as essential fatty acid deficiency observed in CF is not known.

Essential fatty acid status in CF

Low levels of the essential fatty acid (18:2ω6) have been a known complication in cystic fibrosis for about 30 years (Kuo et al., 1962). Many later studies have confirmed this observation (reviewed by Farrell et al., 1985). This abnormality may have important secondary effects such as, growth retardation, changes in cell membrane functions and disturbance of prostaglandin metabolism, and may be related to the underlying causes of some clinical symptoms in cystic fibrosis (Chase et al., 1978; Chase, 1976; Rivers et al., 1975; Hubbard, 1983).

Since recognition of decreased 18:2ω6 in CF plasma and cell membrane, various explanations for the etiology of this biochemical observation have been

proposed. There are many factors that could potentially cause or contribute to decreased linoleate including: 1) malabsorption of ingested fat (Farrell et al., 1985); 2) the traditional tendency of cystic fibrosis patients to restrict dietary fat consumption (Farrell and Hubbard, 1983); 3) greater than normal utilization of dietary polyunsaturated fatty acid for energy production (Hubbard, 1983); 4) increased oxidation of fatty acids due to associated vitamin E deficiency (Chase et al., 1979); 5) liver disease (Caren et al., 1966); 6) a metabolic defect in fatty acid metabolism (Lloyd-Still et al., 1981; Rivers et al., 1975). Of these possibilities, more attention should be focused on whether the deficiency of 18:2ω6 is a primary defect related to the cystic fibrosis gene or just a secondary defect due to malabsorption of fat. Many studies have shown that decreased 18:2ω6 in CF plasma and cell membrane is independent of pancreatic insufficiency and therefore not the result of malnutrition (Chase et al., 1978; Rivers et al., 1975; Rogiers et al., 1982; Rogiers et al., 1983), suggesting that a fundamental defect of fat metabolism is intrinsic and may be due to increased turnover of fatty acid(Lloyd-Still et al., 1981). Whether there is association between the primary defect such as Cl^- impermeability and essential fatty acid metabolism including fatty acid incorporation and desaturation remains unknown.

Cultured Cells in the Study of Fatty Acid Metabolism

The use of cultured cells in place of the intact animal provides an excellent experimental system that has the advantage of being homogeneous and avoids the interferences of many complex physiological interactions with the rest of the body.

Intensive studies on fatty acid metabolism using different cell types including fibroblasts, vascular endothelial cells, platelets, neutrophils, macrophages, hepatoma cells, MDCK cells and cells of neural origin have been thoroughly reviewed (Spector et al., 1981; Rosenthal, 1987). Most cultured cells were found to be capable of taking

up free fatty acid from the culture medium and incorporating it into cellular lipid (Spector et al., 1981). Almost all cultured cells could elongate fatty acid, but not all cultured cells were able to desaturate fatty acid. For example, endothelial cultures, L-fibroblasts, LM cells, HSDM fibrosarcoma and Chang liver cells were reported to have a Δ6-desaturase deficiency (reviewed by Spector, 1981). It was also demonstrated that phospholipid fatty acyl chains of most cultured cells are in a dynamic state and can be modified by medium supplementation (Spector, 1981; Rosenthal, 1987). Furthermore, many different types of cells synthesize cycloxygenase and lipoxygenase derivatives of polyunsaturated fatty acids (Rosenthal, 1987).

Fatty acid metabolism in airway epithelial cells, which line the pulmonary airway and may play a key role in some pathological processes such as inflammation (Holtzman, 1985), asthma (Laitinen et al., 1987) and cystic fibrosis (Drumm et al., 1990), have not received intensive study. For this human cell type the only available information on fatty acid metabolism includes fatty acid composition of both freshly isolated cells and cells cultured in a serum-free condition (Alpert et al., 1991; Holtzman et al., 1986), phospholipid composition (Holtzman et al., 1986) and arachidonic acid (20:4ω6) metabolites (Churchill et al., 1989; Holtzman et al., 1988). The kinetics of incorporation and metabolism of essential fatty acid (18:2ω6) in human airway epithelial cells remains to be investigated.

Nasal epithelium is morphologically and functionally similar to the respiratory epithelium of the lower airways (Yankaskas et al., 1987). Primary cultures of the nasal epithelium retain their normal functions (Bautsch et al., 1988). Thus, nasal turbinates or polyps, available in reasonable amounts from surgery, are useful tissues for study of fatty acid metabolism in human cells.

Platelet Activating Factor

Since fatty acid composition determines membrane structure including the availability of specific phospholipid species and influences the function of membrane proteins such as receptors (Clandinin et al., 1991), it is conceivable that the synthesis and action of cellular mediators, such as platelet activating factor (PAF), that are synthesized from specific membrane phospholipid and act through binding to membrane receptors could be closely associated with membrane fatty acid composition. The biosynthsis, action, biological effects, relation to other lipid mediators and relevance of PAF to cystic fibrosis follows.

Introduction

Platelet activating factor (PAF), one of the most potent autacoids, belongs to a more recently discovered class of mediators with a broad range of biological activities. PAF was first isolated from rabbit blood cells by Benveniste in 1972 and later (1979) identified as a mixture of 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholines having predominantly saturated alkyl moieties with 16 and 18 carbon atoms (Demopoules et al, 1979; Benveniste et al, 1979; Blank et al, 1979). Biochemistry and physiological effects of this potent lipid mediator are described in several review articles (Pinckard et al, 1982; Snyder, 1985; Hanahan, 1986; Braquet et al, 1987; Snyder, 1989; Prescott et al, 1990), the proceedings of three symposia (Benveniste, 1983; Winslow and Lee, 1986; Lipids, 1991) and three monographs (Snyder, 1987; Barnes et al, 1989; Handley et al, 1990). PAF appears to function both in normal physiological events and to mediate pathological responses (Snyder, 1987; Barnes et al, 1989). Clinically, PAF has been suggested to play a role in a variety of clinical conditions such as asthma and pulmonary dysfunction, acute inflammation, transplanted organ rejection, endotoxin and IgE-induced shock, sepsis, thrombosis, cardiac anaphylaxis, gastrointestinal ulceration, allergic skin disease, retinal and

corneal disease, induced liver cirrhosis and ova implantation in pregnancy (Braquet et al, 1987; Snyder, 1987; Barnes et al, 1989; Handley et al, 1990).

Biosynthesis

PAF synthesis has been demonstrated in many cell types including basophils, monocytes, macrophages, neutrophils, platelets and endothelial cells (Braquet et al, 1987; Snyder, 1987). PAF can be formed via remodelling and by de novo biosynthesis (Snyder, 1989; prescott et al, 1990). The remodelling pathway is responsible for synthesis of PAF during stimulus-mediated cell activation, while the de novo pathway appears to maintain the endogenous levels of PAF (Snyder, 1987). The remodelling pathway involves two steps, deacylation and reacetylation. First, the fatty acid residues at the sn-2 position of the alkyl-acyl-glycerophosphocholine precursor, which accounts for 5-50% of total membrane phospholipids (Snyder, 1987), are released by phospholipase A_2 to form alkylyso-glycerophosphocholine (lyso-PAF). The released fatty acid, mostly 20:4ω6, can be converted to eicosanoids (eg. prostaglandins and leukotrienes). Second, acetate is incorporated into the sn-2 position of lyso-PAF by alkylyso-glycerophosphocholine:acetyl-CoA acetyltransferase to yield active PAF (Figure I.4). The acetyltransferase is considered to be a rate-limiting and regulatory enzyme in the biosynthesis of PAF and is activated by stimulus-coupled phosphorylation (Snyder, 1987). De novo biosynthesis consists of three reaction steps starting with alkyllyso-glycerophosphate. Alkyllyso-glycerophosphate:acetyl-CoA acetyltransferase catalyses the conversion of alkyllyso-glycerophosphate to alkylacetyl-glycerophosphate. Then alkylacetyl-glycerophosphate phosphohydrolase hydrolyzes phosphate groups to form alkylacetyl-glycerol. Finally, a dithiothreitol-insensitive alkylacetyl-glycerol:CDP-choline cholinephospho-transferase transfers the phosphocholine moiety to alkylacetyl-glycerol to produce PAF (Figure I.4)). The catabolic pathway for PAF involves removal of the acetate group at the sn-2 position of PAF by acetylhydrolase coupled with a subsequent

acylation of a long-chain fatty acid, primarily arachidonate (Snyder, 1989). Regulation of PAF biosynthesis is still poorly understood.

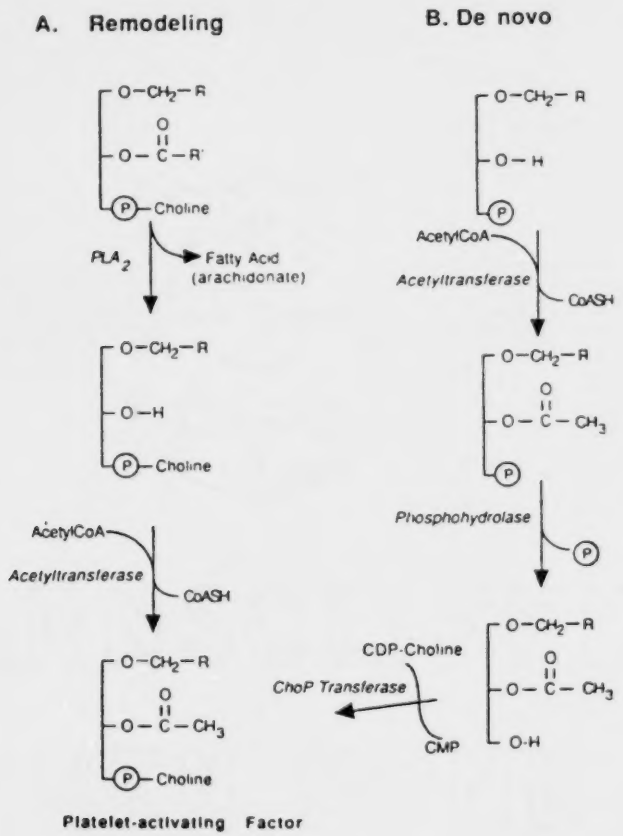

Fig.I.4 Pathways for synthesis of PAF. A, the remodeling pathway begins with the hydrolysis of a fatty acid from the sn-2 position of a choline phosphoglyceride that has an ether-linked fatty alcohol at the sn-1 carbon. This reaction is catalyzed by a phospholipase A_2. The lyso-PAF then is acetylated in a reaction catalyzed by a specific acetyltransferase. B, the de novo pathway starts with an intermediate in the synthesis of ether-linked membrane phospholipids. It is acetylated by a specific acetyltransferase, a different enzyme than in the other pathway. In subsequent steps the phosphate is removed from the sn-3 position and phosphocholine is added. This route is analogous to that for synthesis of bulk choline phosphoglycerides for membranes. The structures shown do not have the correct stereochemistry. (Modified from Prescott et al., 1990)

Biological Importance

PAF is a potent mediator of inflammation (Snyder,1987; Barnes et al, 1989). First, It acts on vessels to induce vasoconstriction or vasodilation, increased vascular permeability and interstitial edema formation; Secondly, PAF recruits and activates platelets, neutrophils and monocytes at the site of an inflammatory reaction; Furthermore, PAF induces the release of other humeral mediators such as eicosanoids (eg prostaglandins, leukotrienes), specific enzymes and oxygen radicals that contribute to tissue injury (Synder, 1987).

PAF affects all major components of the cardiovascular system, including the heart, blood vessels and microcirculation (Feuerstein et al, 1987). One systemic effect of PAF is hypotension. This effect is instantaneous and profound and can be induced with extremely small doses of PAF. PAF may lead to coronary vasoconstriction, ischaemia and low cardiac output and indirectly affects myocardial contractility. PAF induces haemoconcentration and increases permeability of the microcirculation, leading to decreased plasma volume. Moreover, increased levels of PAF have been observed during cardiovascular stress situations such as septic and endotoxic shock, and immune reactions. Thus, PAF may play a central role in the profound cardiovascular derangements that occur in response to acute systemic anaphylaxis (Barnes et al, 1989).

In pulmonary tissue PAF causes airway epithelium damage (Hisamatsu et al, 1991), induces constriction of bronchi (Vargaftig et al, 1980), increases airway microvascular permeability (O'Donnell,1987) and induces a long-lasting non-specific bronchial hyperresponsiveness (Mazzoni et al, 1985; Christman et al, 1987; Chung et al, 1986). PAF also induces a chronic inflammatory response characterized by fibrotic changes in the airway (Handley et al, 1990). In animals PAF elicits acute lung injury characterized by an increased resistance to airflow, pulmonary edema and pulmonary hypertension associated with inflammatory cell recruitment, in particular the eosinophil (Coyle and Page,1990). Thus PAF may be involved in mediating some

pulmonary pathological processes.

In renal pathophysiology PAF induces a dose-dependent decrease in renal blood flow, glomerular filtration rate, urinary flow and sodium excretion. PAF also causes post-glomerular vasodilation, contraction of mesangial cells and increased glomerular permeability with an accumulation of inflammatory cells. Thus PAF may contribute to the clinical-pathological expression of renal disease, such as, endotoxin-induced acute renal failure, glomerular immunopathology and renal allograft rejection (Schlondorff,1990).

PAF has a wide range of deleterious effects on the gastrointestinal tract (Prescott et al, 1990). Parenteral administration of PAF induces vaso-congestion, necrosis and haemorrhagic damage in both the stomach and the small intestine. PAF is also a potent ulcerogen and induces microvascular disturbances and ischaemic episodes and changes in gastrointestinal motility. Thus, the release of PAF makes a major contribution to the associated gastrointestinal damage in endotoxin or septic shock and to the ulcerative process. PAF may be involved in gastritis and esophagitis, as well as inflammation of the small intestine and inflammatory bowel disease (Barnes et al, 1989)

The potential involvement of PAF in physio-pathological conditions has also been demonstrated in many other systems (Barnes et al, 1989). In skin PAF induces both vascular effects, resulting in erythema and plasma protein extravasation relevant to acute inflammatory skin diseases such as urticaria and cellular effects mediating the inflammatory changes associated with chronic skin diseases such as psoriasis. In the nervous system PAF plays a role inducing neuro-degeneration associated with several neuropathological disorders. In the immune system PAF appears to exert several potent immunoregulatory functions at various levels of the immune response. PAF is an essential embryonic mediator for establishment and maintenance of pregnancy and directly affects the foetal lung by stimulating glycogenolysis, thus promoting maturation of foetal lung (Johnston et al, 1987).

Mechanisms of Action

In terms of mechanisms of action of PAF, it is now widely accepted that PAF acts through specific receptors and a variety of signal transduction systems to elicit diverse biochemical responses (reviewed by Chao and Olson, 1993; Shukla, 1992). Specific receptors for PAF have been identified in numerous tissues and cells by a radioligand binding assay (Chao and Olson,1993). The PAF receptor from guinea pig lung was recently cloned by functional expression, showing that the receptor is a G protein-coupled receptor (Honda et al, 1991). Events linking the PAF receptor to the expression of a cellular response are not firmly established, but a general scheme can be offered (Figure I.5). The binding of PAF to its receptor activates GTPase, causes phospholipid turnover via phospholipases A_2, C and D pathways leading to generation of several lipid mediators or second messengers (reviewed by Shulkla,1992; Chao and Olson, 1993) . Activation of phospholipase C hydrolyses phosphatidylinositol 4,5 -biphosphate, yielding diacylglycerol, which activates protein kinase C and inositol triphosphate, which can mobilize subcellular calcium. The attendant rises in cytosolic calcium , some of which may occur via a receptor operated calcium channel, can activate intracellular contractile proteins, phospholipase A2, protein kinase C and perhaps other elements that promote cell function. Protein kinase C may similarly activate regulatory proteins via a phosphorylating mechanism. Activation of phospholipase A_2 mobilizes arachidonic acid which is then rapidly converted to eicosanoids, each of which can modulate cell responses by a receptor mechanism or other mechanisms. Activation of phospholipase D produces phosphatidic acid, which may have a role in the cell and can also be metabolized to generate diacylglycerol. It is also suggested that second messengers generated by these multiple signalling pathways play roles in the PAF-induced expression of primary response genes (Shukla,1992). PAF may serve as a messenger inside the cell and could elicit its actions through interaction with intracullular receptors (Shukla, 1992).

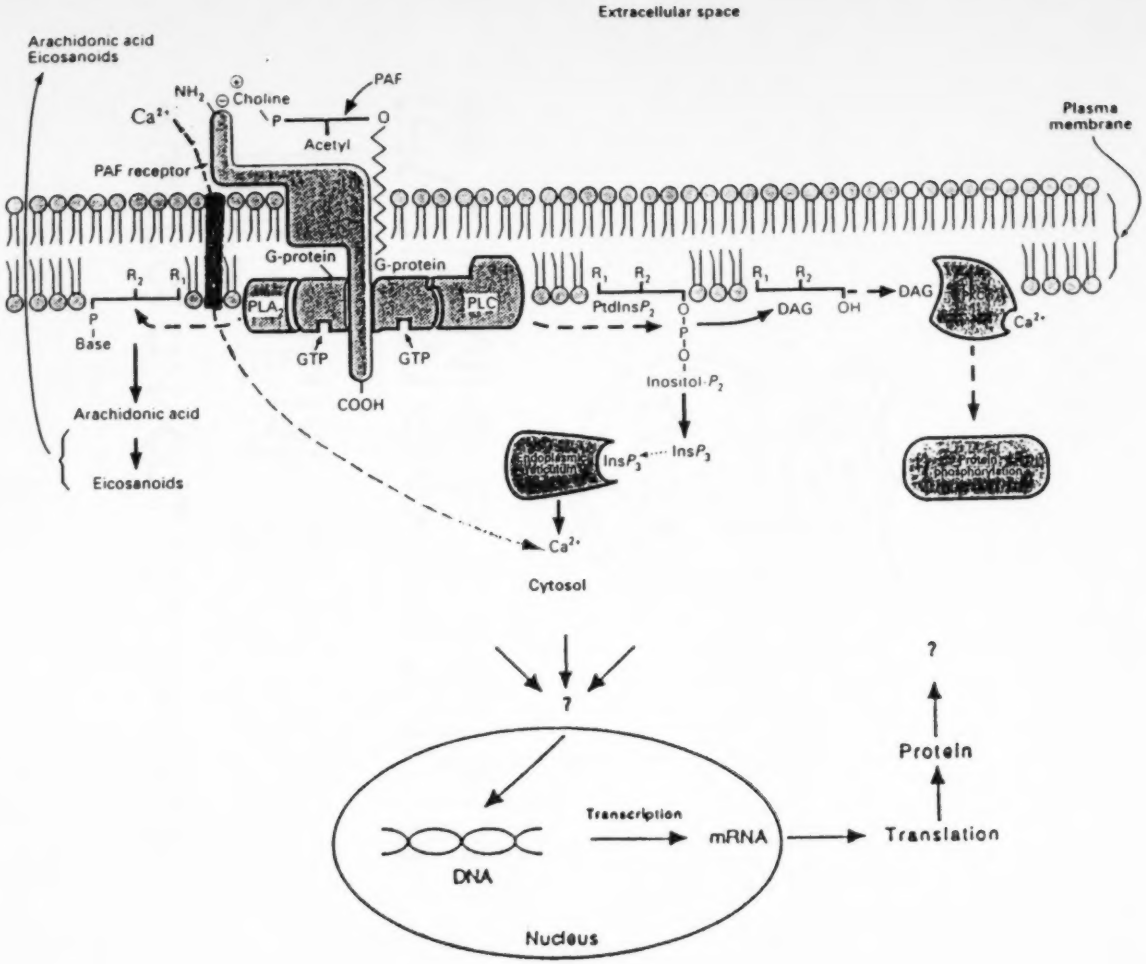

Figure I.5 PAF receptor-mediated intracellular signalling mechanisms. (Modified from Chao and Olson, 1993)

Knowledge of the regulatory factors that affect specific PAF receptors and subsequently control PAF-elicited cellular responses remains in the initial stages of development. It has been found that PAF down-regulates its own receptors, as a result cellular responses such as Ca^{++} mobilization to PAF are desensitized (Shukla, 1992). Although the mechanism for the desensitization remains to be established, this can occur via internalization of the receptor or phosphorylation of the receptor. In neutrophils, specific PAF receptors were modulated by the protein kinase C activator phorbol ester (O'Flaherty et al., 1989; Yamazaki et al., 1989), as well as by LTB_4 (Yamazaki et al., 1989). Cyclic AMP analogues decrease the surface expression of PAF receptors in cultured Kupffer cells (Chao et al., 1990). Bacterial lipopolysaccharide was recently reported to induce an increase in the surface expression of PAF receptors in macrophages (Liu et al., 1992). Numerous PAF receptor antagonists have now been developed by the synthesis of analogs of PAF, screening of natural products and evaluation of a variety of synthetic compounds. PAF antagonists, which have both high affinity and specificity to PAF binding sites, have been demonstrated to antagonize the effect of PAF and thereby prevent or reverse many pathological changes in experimental animals (Barnes et al, 1989; Handley et al, 1990).

It is evident that various monovalent and divalent cations exert regulatory effects on PAF receptors (Hwang et al., 1986). Na^+ and Zn^{++} inhibited [^{3}H]PAF binding while K^+, Mg^{++}, Ca^{++} and Mn^{++} enhanced the specific PAF receptor binding 8-10 fold. The specific mechanism involved in these cation effects on PAF receptors has not been elucidated. Furthermore, Ca^{++} channel blockers and Na^+ channel blockers have been found to inhibit [^{3}H]PAF binding to platelets (Valone, 1987; Hwang, 1989). However, whether Cl^- channel blockers can affect PAF receptor bindingis is not known.

There is little information available so far as to effect of fatty acids on PAF receptor binding. Although two studies have shown that fish oil fatty acid (20:5ω3)

significantly inhibited PAF-induced increase in intracellular Ca^{++} and formation of inositol triphosphate (IP_3) (Bankey et al., 1989; Sperling et al., 1993), how 20:5ω3 induces the effect is not known and the relationship between the inhibition of PAF-mediated responses by 20:5ω3 and effect of 20:5ω3 on PAF binding to its receptor has not been examined.

20:5ω3 has been previously demonstrated to have anti-inflammatory effect (Sperling, 1991). It is possible that inhibition of PAF binding to its receptors and consequently decrease in PAF-elicited cellular responses by 20:5ω3 is a novel mechanism by which 20:5ω3 exerts its anti-inflammatory effect. Therefore, examination of the effect of 20:5ω3 and other fatty acids on PAF binding is needed.

Relationship between PAF and Other Lipid Mediator Systems

A close relationship between PAF and other lipid medators has been suggested (O'Flaherty, 1987; Chilton, 1990). Mammalian cells, when stimulated, mobilize their phospholipids by several concurrently activated pathways (Figure I.6). They release arachidonic acid from the 2 position of cellular phospholipids by a phospholipase A2-mediated reaction (Fig.I.6, pathway B). The arachidonate released can then be metabolized by lipoxygenase-dependent pathways to form eicosatetraenoates (HETEs) or leukotrienes (LTs) or, alternatively, by a cyclooxygense-dependent pathway to form prostaglandins (PGs) and thromboxanes (TXs) (Samuelsson, 1982) (Figure I.7). When phospholipase A_2 acts on alkyl-ether phosphocholines, the product can be acetylated to produce natural PAF (Fig.I.6, pathway A). A final pathway serially phosphorylates phosphatidylinositol (Fig. 1, pathway C). Phospholipase C then cleaves the product to form diacylglycerol and inositol trisphophate; lipase can release arachidonate from the resulting diacylglycerols (Irvine, 1982).

Phospholipid metabolism leads to the formation of many products which are

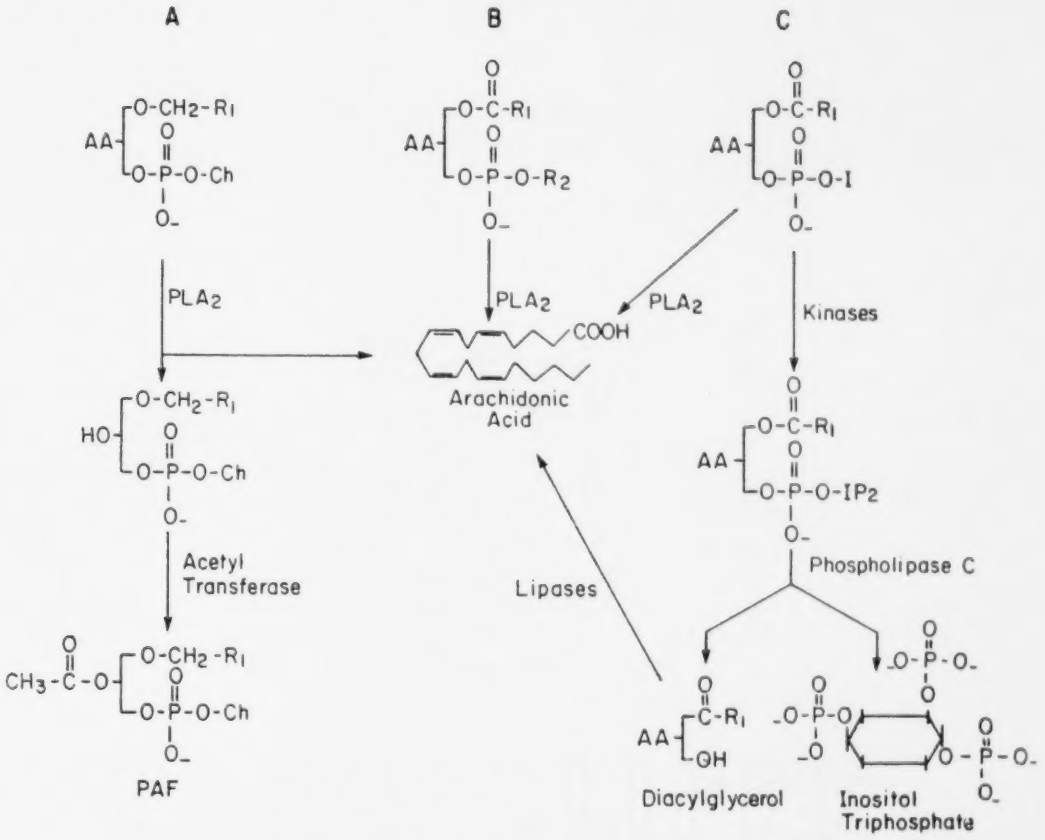

Fig.I.6 Pathways mobilizing cellular glycerolipids. This general scheme illustrates how stimulated cells can (A) produce PAF, (B) directly ralease arachidonate, and (C) metabolized phosphatidylinositols into inositol triphosphate, diacylglycerols, and arachidonate. The extent to which any of these pathways are used depends on the cell type, stimulus, and stimulating conditions studied. R_1: long-chain fatty alkane ; R_2: choline, serine, ethanolamine, inositol, or a proton; AA: arachidonic acid ester; I: inositol; IP_2 inositol diphosphate; PLA_2: phospholipase A_2; Ch: choline. (Modified from O'Flaherty, 1987)

bioactive and may be responsible for mediating ensuing cellular responses (O'Flaherty, 1987). PGG_2, PGH_2, and TXA_2 activate platelets and contract smooth muscle. LTB_4 activates PMN, monocytes, and other granulocytes; it also contracts

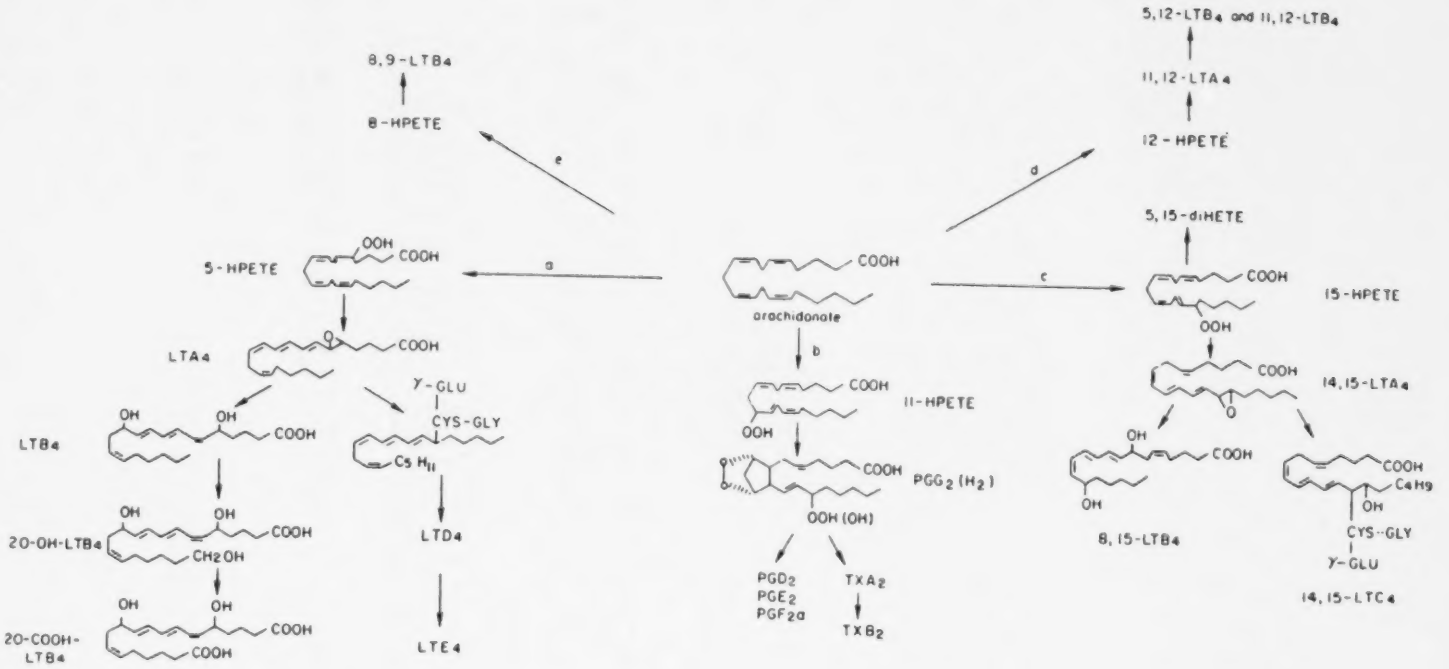

Fig.I.7 Pathways metabolizing arachidonic acid. This general scheme illustrates the pathways producing the hydroxyeicosatetraenoates, leukotrienes, thromboxanes, and prostaglandins. As with glycerolipid turnover, the extent to which any pathway is used is dependent on cell type and stimulus. (Modified from O'Flaherty, 1987)

smooth muscle. LTC_4, LTD_4 and LTE_4 contract smooth muscle and increase vascular permeability. Diacylglycerols activate protein kinase C, and inositol triphosphate mobilizes calcium from endoplasmic reticulum. Each of these products acts by binding to cellular receptors (Lewis et al., 1984).

Both precursors of PAF and eicosanoids, lyso-PAF and arachidonic acid, can be mobilized from a common precursor molecule by the action of a phospholipase A_2 (Figure I.6). It appears that there is interaction between PAF and eicosanoid production. It has been shown that PAF can induce the production and release of arachidonic acid metabolites (Shaw et al., 1981; Chilton et al., 1982; Yousufzai et al., 1985). Conversely, lipoxygenase products have been shown to regulate PAF generation in neutrophils (Billah et al., 1985). Thus mediators have mediators, mediators interact and a coordinate response reflects a complex system of regulatory elements . It remains for future investigations to determine the specific sequence of events involved in the response of a particular cell type or tissue to PAF and eicosanoids.

Potential Relevance of PAF to Cystic Fibrosis

Pulmonary dysfunction represents the most significant clinical component of CF and is the main cause of death in older patients (Hodon et al., 1983). The nature and severity of the bronchopulmonary disease commonly determines prognosis of this disease. The lungs in CF patients are structurally normal at birth but are usually soon affected by recurrent or chronic infection resulting from mucus plugging due to abnormal secretion of bronchial glands and epithelium. The initial pulmonary pathological changes resulting from susceptibility to localized lung infections include bronchiolitis, bronchitis, pneumonia, bronchiectasis, bronchial hyperreactivity (allergy) and mucus hypersecretion. The ensuing cycle of bronchopulmonary infection and mucus overproduction cause characteristic bronchocentric inflammatory and obstructive lesions which eventually lead to the chronic or life-threatening

complications of cor pulmonale, pneumothorax and massive pulmonary hemorrhage (Hodson et al., 1983). Inflammation is the main pathological change and critical factor causing lung damage, thereby pulmonary dysfunction in CF. Thus inflammatory mediators play a key role in this pathological process.

PAF is a potent inflammatory mediator acting by 1) increasing vascular permeability; and 2) recruiting and activating inflammatory cells in local infection sites, which leads to interstitial edema and tissue damage. PAF also induces the release of other inflammatory mediators (eg. eicosanoids) that contribute to tissue injury. Based on animal experiments, PAF can directly damage airway epithelium, inducing constriction of bronchi and causing pulmonary fibrosis, resulting in bronchial hyper-responsiveness, pulmonary hypertension and pulmonary edema (Synder, 1987; Barnes et al., 1989) Furthermore, previous studies have found that higher than normal levels of PAF are present in bronchial sputum of CF patients (Arnoux et al., 1988) and that CF cells have higher activity of phospholipase A_2 (Standvik, 1989), which is a key enzyme for PAF synthesis. Thus, PAF may play an important role in the pathogenesis of pulmonary disease in CF. A new strategy in the treatment of pulmonary disease in CF may ensue via inhibition of PAF generation or of the action of PAF.

Chapter II

RESEARCH PLAN

Rationale

Epithelial cells lining the pulmonary airway may play an active role in initiating the inflammatory response to inhaled stimuli. The functions of these cells depend to some extent on properties of membrane lipids and production of lipid mediators. The capacity of epithelial cells to incorporate and desaturate 18:2ω6 to produce 20:4ω6 plays a key role in regulation of membrane properties and functions of human airway epithelium. However, whether or not human airway epithelial cells possess the ability to incorporate and desaturate essential fatty acid (18:2ω6) and whether the fatty acid composition of the cells can be readily modified by exogenous fatty acids is not known. Thus, understanding and characterization of 18:2ω6 metabolism in human airway epithelial cells is of basic as well as clinical importance.

In cystic fibrosis, both chloride ion impermeability across epithelial cell membrane and low levels of 18:2ω6 in many tissues and plasma is known. However, the cause of the low levels of 18:2ω6 in CF is still controversial. Although many studies have suggested that a defect in metabolism of essential fatty acid may be responsible for the abnormality, it is unknown whether impermeability of Cl^- affects incorporation and metabolism of 18:2ω6 in airway epithelium. Therefore, examination of the effect of inhibition of Cl^- conductance on 18:2ω6 incorporation into cellular lipids could provide better understanding of the apparent essential fatty acid deficiency in cystic fibrosis.

Platelet activating factor (PAF), generated from a membrane phospholipid and acting through interaction with membrane-associated receptors, is the most potent

lipid mediator of inflammation and has been implicated in the pathogenesis of pulmonary infection, the main clinical manifestation of cystic fibrosis (CF). Understanding the biosynthesis and action of PAF in pulmonary tissues may contribute to new methods of modulating pulmonary inflammatory process in CF as well as asthma. However, regulatory factors affecting PAF production and action in airway epithelial cells have not been elucidated. Thus study of these areas, particularly the effect of membrane fatty acid composition on the generation and action of PAF, is merited.

Research Objectives

The objectives of this thesis research are:

1. To characterize essential fatty acid incorporation and desaturation in human airway epithelial cells.
2. To define the turnover rate for phospholipid fatty acyl chains and change in fatty acid composition of the epithelial cell membrane lipid by exogenous fatty acids.
3. To examine the effect of a chloride-channel blocker on fatty acid incorporation and desaturation in the epithelial cells.
4. To identify the PAF receptor in human airway epithelial cells and to determine the effects of fatty acids and ion channel blockers on PAF receptor binding activity.
5. To examine the effect of exogenous fatty acids on generation of PAF and interaction between PAF and eicosanoid production.

Hypotheses

It is specifically hypothesized that in human airway epithelial cells:

1. Exogenous fatty acids can be incorporated into the epithelial phospholipids and triglyceride.

2. 18:2ω6 can be desaturated and elongated to 20:4ω6.
3. Turnover rates of individual phospholipid fatty acyl chains are different.
4. Supplementaion of the epithelial cells with different fatty acids results in dynamic alteration in fatty acid composition of membrane phospholipid.
5. Application of a Cl^- channel blocker to the cultured cell alters essential fatty acid perhaps by reducing incorporation into phospholipid and/or desaturase activity.
6. Human airway epithelial cells express functional receptors for PAF.
7. Exogenous fatty acids, particularly 20:5ω3, can alter PAF binding to the cells.
8. Alteration of PAF binding by 20:5ω3 results in changing PAF receptor-mediated cellular responses (e.g. calcium mobilization).
9. Calcium, sodium and chloride ion-channel blockers may affect PAF binding activity.
10. Human lung epithelial cells can produce PAF upon stimulation; production of PAF can be modulated by supplementation of the cells with different fatty acids.
11. Eicosanoids can induce PAF synthesis in epithelial cells.

Chapter Format

The hypotheses posed were tested in a sequence of experiments. These experiments are organized in chapters as follows:

Chapter III describe all the methods used in the subsequent chapters.

Chapter IV examines 1) the capacity of human airway epithelial cells to

incorporate and desaturate 18:2ω6 (hypotheses 1 and 2); 2) turnover rate for phospholipid fatty acyl chains and effect of fatty acid supplementation on membrane lipid composition (hypotheses 3 and 4). Epithelial cells isolated from human nasal polyps were cultured for 5-7 days, and then incubated with radiolabelled [1-^{14}C]-18:2ω6. After incubation, cells were harvested and lipids were extracted and separated on TLC. The radioactivity in lipid fractions and individual fatty acids was determined. The turnover rate was assayed by pulse-chase studies in which the cultured cells were pulsed with ^{14}C-18:2ω6 for 2 h followed by a chase with 18:2ω6 for 1-4 h. After the chases at different times, change in labelled fatty acids in the medium and cellular lipid fractions was determined. The half-life was calculated according to the change in label present in individual phospholipid. Fatty acid composition of membrane phospholipid from the cells incubated with or without 18:2ω6 supplementation was analyzed by gas chromatography.

Chapter V examines the effect of a Cl^- channel blocker, anthracene 9-carboxylate (9-AC) on incorporation and desaturation of fatty acids (hypothesis 5). 9-AC dissolved in dimethyl sulfoxide was added to the culture medium. After 5 min incubation with the cells, labelled fatty acid (^{14}C18:2(6)) was added and incubated for various times. After incubation, the labelled fatty acids in the cells were analyzed. The effect of 9-AC was determined by comparison with a control sample without addition of 9-AC.

Chapter VI examines 1) characterization of [^{3}H]PAF binding to human airway epithelial cells (hypothesis 6); 2) effect of exogenous fatty acids on PAF binding and PAF-induced Ca^{++} mobilization (hypothesis 7 & 8); 3) effect of Ca^{++}, Na^+ and Cl^- channel blockers on PAF binding to the epithelial cells (hypothesis 9). Specific PAF binding site was determined by radioligand binding assay in which a cell suspension

was incubated with [^{3}H]PAF with or without unlabelled PAF at 4°C. Binding affinity and number of PAF receptor were measured by Scatchard analysis. Function of the receptors was tested by measuring response of [Ca^{++}]i to PAF. To test the effect of fatty acid supplementation on PAF binding, 100 μM of different fatty acids was added to culture medium and incubated with cells for 6 h, then membrane fatty acid composition, specific PAF binding and intracellular Ca^{++} concentration were examined. To test the effect of ion channel blockers on PAF binding, epithelial cells were first incubated with various ion channel blockers at 37°C for 15 minutes, then specific binding of PAF to the cells was determined by radioligand binding assay.

Chapter VII examines effects of membrane fatty acid composition and eicosanoids on PAF production (hypothesis 10 & 11). Fatty acids bound to BSA were added to medium and incubated with cells for 6 h. The cells were stimulated with either calcium ionophore A23187 or eicosanoids, then PAF was detected by radioimmunoassay.

A general summary and discussion is provided in **Chapter VIII.**

Chapter IV - VII have been presented in paper format as follows:

Chapter IV:
Kang, J.X., Man, S.F.P., Brown, N., Labrecque, P. and Clandinin M.T. (1992) Essential fatty acid metabolism in cultured human airway epithelial cells. Biochim. Biophys. Acta 1128: 267-274.

Chapter V:
Kang, J.X., Man, S.F.P., Brown, N., Labrecque, P. and Clandinin, M.T. (1992) The chloride channel blocker anthrancene-9-carboxylate inhibites fatty acid incorporation into phospholipid in human airway epithelial cells. Biochem. J. 285(3): 725-729.

(Parts of data from Chapter IV and V have also been presented in)
Kang, J.X., Man, S.F.P., Brown, N., Labrecque, P. and Clandinin, M.T. (1992) Essential fatty acid metabolism in cultured human airway epithelial cells: Effect of choloride channel blockers. In Essential Fatty Acids and Eicosanoids (Gibson, B. and Sinclair, A., eds.) AOCS, Champaign, Illinois. PP.453-455.

Chapter VI:
Kang, J.X., Hirsh, A.J., Man, S.F.P., and Clandinin M.T. (1993) Characterization of platelet activating factor binding to cultured human airway epithelial cells: modulation by fatty acids and ion channel blockers (Submitted to Biochem. J.)

Chapter VII:
Kang, J.X., Man, S.F.P., Brown, N. and Clandinin, M.T. (1993) Modulation of platelet activating factor generation by modification of membrane fatty acid composition in a human lung epithelial cell line (A549). (Submitted to Journal of Lipid Mediator)

Chapter III

METHODS

This chapter describes all the methods or experimental procedures used in subsequent chapters.

This research was approved by the Research Ethics Board of the Faculty of Medicine, University of Alberta.

Patients and Tissue Origin

Tissues were obtained from normal subjects who underwent excision of turbinate epithelium (or nasal polyp) for reconstructive consideration or relief of airway obstruction. The subjects had a mean age of 37.9 ± 10.9 yr with a range of 14 to 62 yr. Subjects were not taking regular or chronic medication.

Cell Isolation

Human nasal turbinates, received 1-4 hours following surgery, were placed in Jokilks modified minimum essential medium (MEM) supplemented with antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin sulphate and 500 μg/ml gentamycin) at 0°C for transport from surgery to the laboratory. Cartilage and excess submucosal tissue were dissected free and the epithelial sheets were transferred to a solution of 1 mg/ml type 14 protease in MEM with the same antibiotics and digested overnight at 4°C with mild agitation. After raising the temperature to 37°C for 1-2 hours in a 5% CO_2 incubator, the cells were separated from stroma by mild hand agitation for 1 min. The cell suspension was collected and 10% (v/v) fetal bovine serum (FBS) added to neutralize the protease. The cells were filtered through a 60-μm Nitex mesh to remove debris, centrifuged at 1300 rpm for 10 min and washed once in 10% FBS-

DME/F_{12}. The resultant cell pellets were suspended in culture medium (see below), before plating on culture plates.

Primary Cell Culture

Epithelial cell suspension was plated on six small collagen-sheet plates with culture medium (DME/F_{12}, hormones, antibiotics, and 1% FBS). The medium supplements were: insulin, 2 μg/ml; transferrin, 7.5 μg/ml; endothelial cell growth factor, 7.5 μg/ml; epidermal cell growth factor, 18 ng/ml; hydrocortisone, 36 mg/ml; triiodo-L-thyronine (T_3), 2 ng/ml; cholera toxin, 10 ng/ml; penicillin G, 60 μg/ml; streptomycin sulfate, 100 μg/ml; gentamycin, 50 μg/ml. The cells were cultured at 37°C, in 5% CO_2, 95% air, with 98% relative humidity in a tissue culture incubator (Model 3173; Forma Scientific) and the growth medium was changed every other day. After 5-7 days culture, the cells were confluent, then harvested and used for incorporation and metabolism or PAF binding studies.

The cells were detached from the plate surface with the aid of 0.05% trypsin/EGTA solution (after removing culture medium, 1 ml of trypsin/EGTA solution was added to each well and incubated for 5-10 min at 37°C). After neutralization with FBS and washing, the cell pellets were suspended in fresh culture medium and counted in a hemocytometer (normally 3-5 x 10^6 cells/culture) with a epithelial purity of more than 90%. Identification of epithelial cells by cytokeratin stain is shown in Figure III.1. Cell viability was more than 95% by trypan blue exclusion.

Cell line Culture

Human bronchical epithelial cells (ATCC-CC-185,A549) were obtained from the American Type Culture Collection (Rockville, MD). These cells were originally derived from human carinoma and found to biochemically and morphologically

A

B

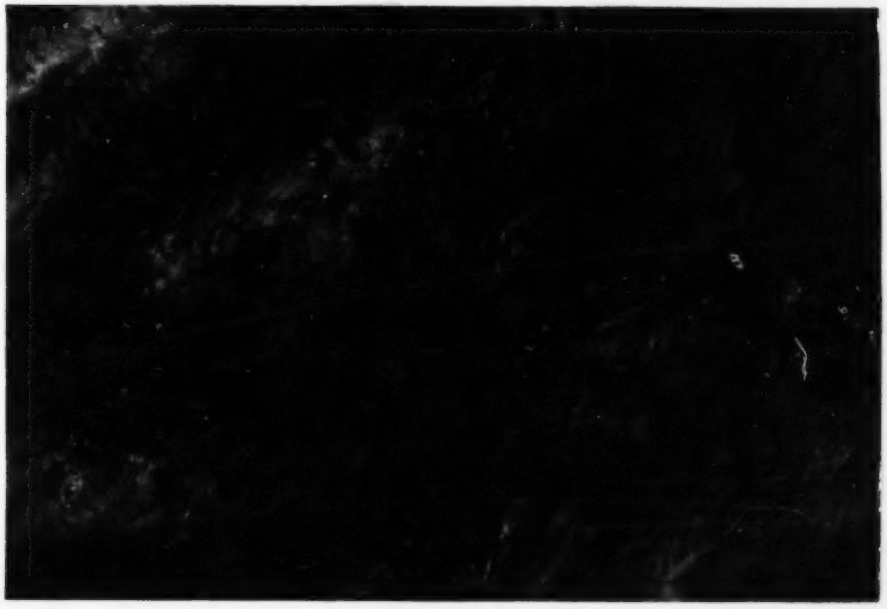

Figure III.1 Photographs of human nasal epithelial cells in primary culture (A) Bright field view of epithelial cells. (B) Immunohistochemical staining of epithelial cells for keratin.

resemble type II epithelial cells (lieber et al.,1976). Cells were grown in Ham's F_{12} medium plus penicillin and streptomycin supplemented with 10% fetal bovine serum in a 37°C, 5% CO_2, 98% relative humidity incubator (Model 3137, Forma Scientific). Medium was changed 2-3 times weekly until a confluent monolayer was formed.

Modification of cellular fatty acid composition

To modify membrane fatty acid profile, various fatty acids were suspended by sonication at 37°C in sterile 5% (w/v) BSA, then 100 μl volume was added to serum-free culture medium to give a final concentration of 100 μM. After 6 h incubation with fatty acid the cells were harvested and subjected to fatty acid analysis as described below. Cell viability was not affected by this incubation with fatty acids.

Isotope Incubation

Primary cultured cells were harvested and washed once with culture medium , then an aliquot was removed for analysis of protein and lipid. The remaining cells were equally replated on plastic culture dishes (5-8 x 10^5 cells/dish) with 2 ml of culture medium (without FBS). Mixtures of [1-^{14}C] -labelled fatty acids and unlabelled fatty acid, suspended by sonication at 37°C in sterile 5% bovine serum albumin, were added in a 100 μl volume to a final fatty acid concentration of 10 μM and 2.2x10^6 dpm of ^{14}-C-labelled fatty acid and 40 μM for unlabelled fatty acid (2.2x10^4 dpm/nmole fatty acid) (unless otherwise specified). Cells were incubated for various times at 37°C, then harvested, washed with phosphate-buffered saline (PBS) and centrifuged. The cell pellets and culture medium plus washed buffer were extracted (see Lipid Analysis for details) and used for all determinations (incorporation and/or desaturation of labelled fatty acids).

To study the effect of ion-channel blockers, anthracene-9-carboxylate was added to the cells 5 min before fatty acids were added. Anthracene-9-carboxylate was

dissolved with dimethyl sulphoxide (DMSO) in final concentration of 0.5%. At this concentration, DMSO alone did not interfere with fatty acid metabolism.

For experiments using CI-free solutions, culture medium was replaced by buffer solution containing 1.2 mM $CaSO_4$, 1.2 mM$MgSO_4$, 118.9 mM Na-gluconate (NaCI for control), 20.4 mM $NaHCO_3$, 2.4 mM K_2HPO_4, and 0.6 mM KH_2PO_4. The cells were incubated in this solution with fatty acids for only 2 hours.

For turnover studies, cells were pulse-labelled with [1-^{14}C]-labelled fatty acid (18:2ω6) (1 μCi/dish, 30 μM) for 2 h. After incubation, the radioactive medium was removed. Cells were washed once with 0.5% bovine serum albumin in phosphate buffered saline and were further incubated with fresh medium containing 100 μM unlabelled 18:2ω6 for various times. After the chase, cells were harvested and the medium was removed by centrifugation. Cells were washed once and subjected to lipid analysis.

Lipid Extraction

Cell (or medium) lipids were extracted by the following procedures: chloroform:methanol (2:1, v/v) (Folch, 1957) containing 0.005% butylated hydroxytoluene (as antioxidant) was added and mixed vigorously for 1 min then left at 4°C overnight. 1 ml (or 5 ml for medium) of 0.88% NaCl was added and mixed again. The chloroform phase containing lipids was collected. The remains were extracted twice with 2 ml (5 ml for medium) chloroform. The chloroform was pooled and dried under nitrogen and stored in sealed tubes at -70°C. Recovery rate of labelled fatty acid was >95%.

Thin Layer Chromatography (TLC)

TLC plates were activated at 100°C for 60 minutes. TLC tanks were equilibrated with solvent by lining the tank with Whatman #1 filter paper and

allowing the tank to stand at least 1 hr prior to inserting plates. Lipid was visualized under ultraviolet light after spraying the plate with 0.1% (w/v) 2'7'-DCF in 95% (v/v) MeOH or 0.01% (w/v) ANSA in water.

Phospholipids were separated from neutral lipids by running the sample on Analtech 250 μ Silica Gel G-plates (20x20cm) in petroleum : diethyl ether : acetic acid (80:20:1, v/v/v) for ~-35 minutes (Skipski et al., 1969). Individual phospholipids were separated on Analtech 250 μ Silicon Gel H-plates (20x20 cm) using the following solvent system: chloroform : methanol : 2-propanol : 0.25% (w/v) KCl : triethylamine (30:9:25:6:18 by vol.)(Touchstone et al., 1980). The plates were developed for 90 min.

Fatty acid methyl esters were separated according to degree of unsaturation on Analtech 250 μ Silica Gel H-plates impregnated with $AgNO_3$ to a final concentration of 30% (w/w) $AgNO_3$ in silica. Carrier methyl esters of fatty acids (18:1ω9, 18:2ω6, 20:3ω6, 20:4ω6) were spotted with the labelled samples. Plates were developed first in hexane : diethyl ether : acetic acid (94:4:2 by vol.) for 35 min and then in hexane : diethyl ether : acetic acid (90:10:2 by vol.) for 25 min as sequential solvent systems. Bands containing monoenoic, dienoic, trienoic, and tetraenoic fatty acids could be identified by comparison with individual reference standards.

Liquid-Scintillation Counting

All [^{14}C]-labelled lipids separated by TLC were scraped directly from plates into 5 ml plastic scintillation vials (Fisher minivials) containing 5 ml of scintillation cocktail (Aquasol, NEN or scintiverse, Fisher). Samples were counted in a Beckman LS-5801 Liquid Scintillation Counter. Counting efficiency was 90-97% and 50-55% for ^{14}C and ^{3}H respectively. Quench was monitored by "H-number" method. Radioactivity was expressed as dpm before molar equivalents were calculated.

Formation of Fatty Acid Methyl Esters

Fatty acid methyl esters were prepared using BF_3/methanol reagent (Morrison and Smith, 1961). Sample containing lipid in silica was added directly to 2 ml hexane in 16 ml glass tubes with teflon-lined caps. BF_3/MeOH reagent (1.5 ml) was added, samples were heated at 90-110°C in a sand bath for 1 hour, cooled to room temperature and methyl esters extracted in the hexane phase after addition of 1 ml H_2O. Samples were allowed to stand for 20-30 minutes, then the upper hexane layer was removed and concentrated under nitrogen.

Gas-Liquid Chromatography

Fatty acid methyl esters were separated by gas-liquid chromatography using a fully automated Varian Vista 6000 GLC equipped with a flame-ionization detector (Hargreaves and Clandinin, 1987). The chromatography utilized a fused silica BP_{20} capillary column (25m x 0.25 mm I.D.). Helium was used as the carrier gas at a flow rate of 1.8 ml/min using a splitless injection. The initial oven temperature was 150°C, increased to 190°C at 20°C/min and held for 23 min, then increased to 220°C at 2°C/min for a total analysis time of 40 min. These analytical conditions separated all saturated, mono- and polyunsaturated fatty acids from C_{14} to C_{24} carbons in chain length. A Varian Vista 654 data system was used to analyze area percent for all resolved peaks and to quantify sample size based on external standards when added.

Phosphorus Determination

The identified phospholipids were scraped into phosphorus-free test tubes from TLC plates. Phosphorus was determined by a modified Bartlett assay for micro-phosphorus analysis (Marinette, 1961). The method involved the following in sequence: digestion of the sample in 0.9 ml of 70% $HClO_4$ at 160-180°C (tubes covered with marble) for 39 min, air cooling, addition of 7.0 ml of distilled water, 1.5

ml of 25% ammonium molybdate, and 0.2 ml of ANSA reagent (described by Bartlett, 1959), mixing and heating for 7-8 min in a boiling water (tubes covered with marble), then cooling for 20-30 min. The intensity of the blue colour produced was measured at 830 nM on a Beckman Spectrophotometer. Appropriate blanks (Silica only) and standards were also run. Standard solution of Pi containing 1 mg Pi/ml was prepared by dissolving 439 mg of KH_2PO_4 in 100 ml of distilled water. A standard curve was prepared using 0.5-10 μg phosphorus.

Protein Determination

Protein content of cells was measured by a colorimetric method (Lowry et al., 1951). Reagent A contained: 2% $CuSO_4$: 4% Na^+-K^+-tartrate: 3% Na_2CO_3 : 0.1 N NaOH (1:1:48 by vol); Reagent B contained: phenol reagent: 20 (1:2 by vol). Standards and samples were mixed with 5 ml reagent A, standed at room temperature for 10 min and read at 660 nM in a Beckman Spectrophotometer against a prepared blank. A standard curve was prepared using 1-100 μg bovine serum albumin. Cells contained approximately 0.8-1.0 mg of protein/1 x 10^6 cells.

[^{3}H]PAF binding assay

In light of the fact that most receptors on the epithelial cell are situated in the basolateral membrane (Nelson, 1991), PAF binding assay was performed using cell suspensions. Primary cultured cells were detached with 0.05% trypsin/EGTA, washed twice with 10% (v/v) FBS medium and incubated at 37°C for at least 1 h before they were used for binding. Viability of the cells was more than 95% as measured by trypan blue exclusion. The binding of PAF in the cell suspension, when compared with the binding measured initially in monolayer as well as in the cells harvested by scraping was more reproducible with smaller variations and showed higher levels of binding, suggesting that mild trypsinization did not affect PAF binding activity.

Cells (1-2 x 10^{6}) were resuspended in 1 ml of HBSS containing 10 mM HEPES and 0.25% (w/v) BSA was added to 1.5 ml microcentrifuge tubes. Cell suspensions were incubated in duplicate with [^{3}H]PAF with or without unlabelled PAF or WEB 2086 at 4°C for appropriate times. This temperature (4°C) was used to minimize both the incorporation into cells and the metabolism of [^{3}H]PAF. The cells were harvested by centrifugation for 1 minute in a Beckman microfuge (Irving, CA). The supernatant was carefully aspirated and the cell pellet was washed twice with 1 ml of cold HBSS containing 0.25% BSA. The resulting cell pellet was resuspended in 300 μl of 10% (w/v) sodium dodecyl sulfate, mixed with 10 ml of scintillation cocktail (ScintiVerse, Fisher) and assessed for cell-bound radioactivity in a Beckman LS5801 liquid scintillation counter. Non-specific binding was defined as total binding in the presence of 1000-fold excess unlabelled PAF. Specific binding was calculated as the difference between total and non-specific binding. The dissociation constant (Kd) and the maximal binding (Bmax) was determined by using Scatchard analysis (Scatchard, 1949).

Intracellular calcium measurement

Intracellular free calcium levels were monitored by methods similar to those described by Merritt, et al (1990). Isolated epithelial cells were loaded with Fluo-3 by incubating the cell suspension in the buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes and 10 mM glucose, pH 7.4) with (5 μM) Fluo-3/AM at 37°C for 40 min. The cells (1x10^{6} cells/ml) were then washed once with buffer and resuspended in buffer supplemented with 1 mM $CaCl_2$ and 0.5% (w/v) BSA. An aliquot (0.5 ml) of cells was transferred to a 1 ml fluorometer cuvette and prewarmed to 37°C. Cell fluorescence was monitored at 37°C with constant mixing in a CAF-100 Ca^{++} Analyzer (JASCO) at 505 nm excitation, 530 nm emission. WEB 2086 was introduced with (<0.5%) ethanol as the solvent which did not affect baseline $[Ca^{++}]i$. $[Ca^{++}]i$ was calculated using the following equation: $[Ca^{++}]i = K \times \{(F -$

Fmin)/(Fmax - F)}, where K is the dissociation constant of Fluo-3-Ca^{++} and is 864 nM at 37°C , F is the Fluo-3 fluorescence measured during the experiments. Fmax and Fmin are the fluorescence when Fluo-3 is calcium-saturated and calcium-free, respectively. Fmax was determined by lysis of the cells with digitonin at the end of each experiment. Fmin was obtained by addition of enough EGTA following lysis of the cells .

Radioimmunoassay for PAF

After 6h incubation of cells with fatty acid or vehicle (albumin), the culture medium was removed. Cells ($1x10^6$) were resuspended in 1 ml of Hanks balanced salts solution (HBSS) containing 0.25% bovine serum albumin (BSA) and incubated with and without 2.5μM of ionophore A23187 or 3μM of eiconsanoids (PGE_2 , $PGF_{2\alpha}$, LTB_4 , LTD_4 or LTE_4) for 10 min at 37°C. Incubation was terminated by addition of 2.6 ml solution of methanol/chloroform (2:1). Lipids were immediately extracted by the method of Bligh and Dyer (1959). The lower chloroform phases were collected and dried under nitrogen. The PAF-containing residues were reconsitituted in 400 μl of assay buffer and 100 μl volumes were taken for radioimmunoassay. The PAF concentrations were assayed using PAF [^{3}H] scintillation proximity assay (SPA) Kit (TRK-990) purchased from Amersham (London). This assay system utilizes the novel technique of scintillation proximity assay , which eliminates the need to separate antibody bound from free ligand, common to heterogeneous methods. The system combines the use of a high specific activity tritiated PAF tracer with an antibody specific for PAF and a PAF standard. The sensitivity of the assay is appox 20 pg/tube. Cross-reactivity with lyso-PAF is <0.01% and with phosphatidylcholine is <0.04%. Performance of the assay and calculation of results were according to the manufacturer's recommendations. All extractions, sample handling and the radioimmunoassays were performed in polypropylene glassware.

Statistical Analysis

Results are presented as means ± SD. Comparison between individual groups was made by using Student T-test (Steele and Torrie, 1980) and a P level < 0.05 was considered statistically significant. Regression analysis was used to determine the relationship between age and desaturase activity. Receptor binding data was analyzed by Scatchard analysis.

Chapter IV

ESSENTIAL FATTY ACID METABOLISM IN CULTURED HUMAN AIRWAY EPITHELIAL CELLS

Introduction

Changes in the composition of membrane phospholipid fatty acid chains can affect a number of cellular functions, including ion transport and transmenbrane signal transduction, by altering the confirmation, activity and translational diffusion of proteins embedded in the lipid matrix (Quinn, 1981; Stubbs, 1984; Clandinin et al., 1985). Relative availability of saturated and unsaturated fatty acids for phospholipid synthesis is determined by diet, fatty acid incorporation and activity of elongase and desaturase enzymes. Thus the capacity of a particular cellular or subcellular fraction to elongate-desaturate available fatty acid substrate, and deacylate-reacylate existing membrane lipid fatty acids will determine in situ control of membrane phospholipid composition.

Δ^6-Desaturase converts 18:2ω6 to 18:3ω6 and is thought to be a rate-limiting step in the synthesis of arachidonic acid (20:4ω6) from 18:2ω6 (Walkle, 1983; Brenner, 1984). 20:4ω6 is generally present in the sn-2 position of membrane phospholipid, plays an important role for membrane physico-chemical properties and is a common precursor for synthesis of eicosanoids (prostaglandins, leukotrienes and thromboxanes). In the airway epithelium, prostaglandin production has been implicated as a mediator of ion transport, particularly Cl^- secretion (Leikauf, 1985; Eling, 1986; Liedtke, 1986). Thus, whether or not the airway epithelium can utilize 18:2ω6 and synthesize 20:4ω6 is critical for a variety of cell functions in this cell type.

Δ^6-Desaturase activity is present in some but not all tissues and cultured cells of mammalian origin (Dunbar, 1975; Maeda, 1978) and within a given species, expression of Δ^6-desaturase activity may vary. For example, Δ^6-desaturase activity is present in human lung fibroblasts (Dunbar, 1975; Maeda, 1978) but is evidently absent in cultured endothelial cells from human umbilical vein, which are incapable of converting ^{14}C-linoleic acid to arachidonic acid (Spector, 1981). Furthermore, Aplert et al. (1991) recently postulated, in light of the low level of 20:4ω6 found in cultured human airway epithelial cells, that these cultured cells may lack Δ^6-desaturase. Should a deficiency in Δ^6-desaturase activity in fact exist in human airway epithelial cells, it would imply that not only do these cells require a source of essential fatty acids, but such cells might also be dependent on an exogenouos source of 20:4ω6. Whether human airway epithelial cells lack Δ^6-desaturase and/or Δ^5-desaturase activity remains to be determined by direct experiments with appropriate precursor fatty acids. Moreover, incorporation of essential fatty acid into these cell membrane lipids must be characterized. Understanding airway epithelial lipid metabolism may be of clinical importance in terms of dietary fat supplementation to patients with pulmonary diseases such as cyctic fibrosis.

The objective of the present study is to characterize incorporation and desaturation of 18:2ω6 in cultured human airway epithelial cells, to assess the turnover rate of this fatty acid in epithelial phopholipids and to determine the effect of fatty acid supplementation on fatty acid composition of membrane phospholipid.

Methods

The methods used in this study include cell isolation, primary cell culture, isotope incubation, lipid extraction, thin layer chromatography, liquid scintillation counting, formation of fatty acid methyl esters, gas-liquid chromatography, phosphorus and protein determination, which are described in detail in Chapter III.

Results

Incorporation of labelled fatty acid in cells

Cultured human airway epithelial cells efficiently incorporated essential fatty acid (18:2ω6) and its desaturaed products into both the neutral lipid and phospholipid of the cell. Following 4 h incubation the total amount of [1-C^{14}]-18:2ω6 incorporated into the cellular lipids was 32 ± 5.6 nmole per mg protein of cultured cells with 65 ± 9.5% in phospholipid and 31 ± 10% in neutral lipid. Fatty acid incorporation into cell membrane was time-dependent (Figure IV.1). Within 4 h [1-

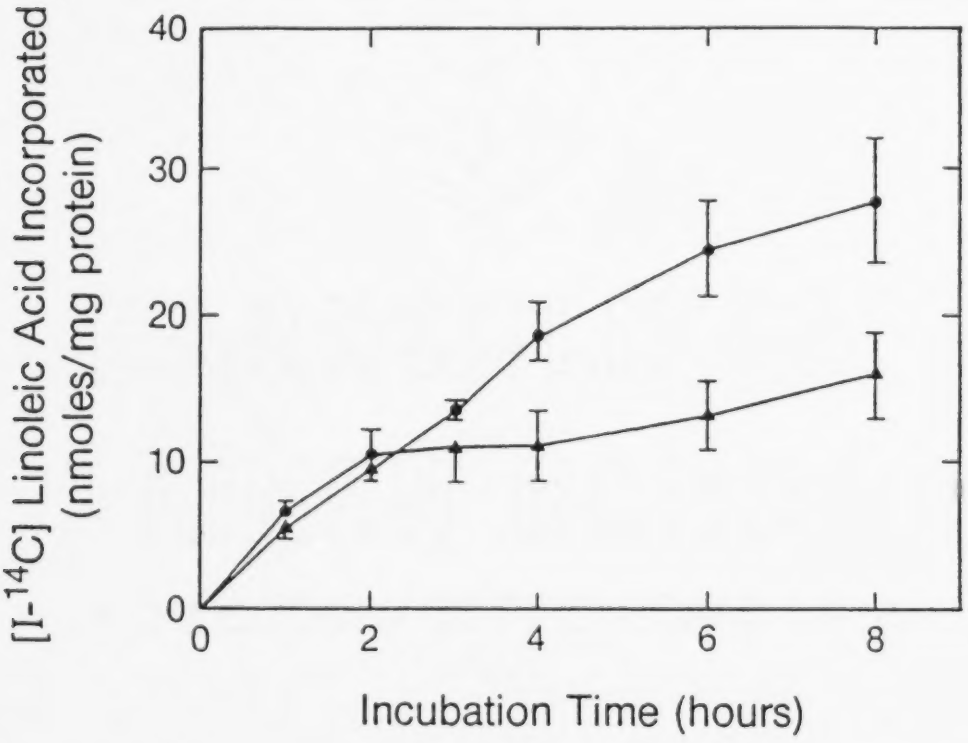

Figure IV.1 Time course of [1-^{14}C]-18:2(6) incorporation into total phospholipid (●-●) and tri –glyceride (▲-▲) of human airway epithelial cells.
Cells were incubated with 1 μCi [1-^{14}C]-18:2(6) (100 nmoles) for various times as indicated. Lipid were extracted, separated on TLC plates and counted with a scintillation counter. Values are means±SD of 4 experiments.

^{14}C]-18:2ω6 incorporation into phospholipid increased linearly, followed by a relatively slower increase from 4 h to 8 h. During the first 2 h the amount of fatty acid incorporation into triglyceride was the same as that incorporated into phospholipid. After 2 h the rate of [1-^{14}C]-18:2ω6 incorporation into triglyceride was very low (Figure IV.1).

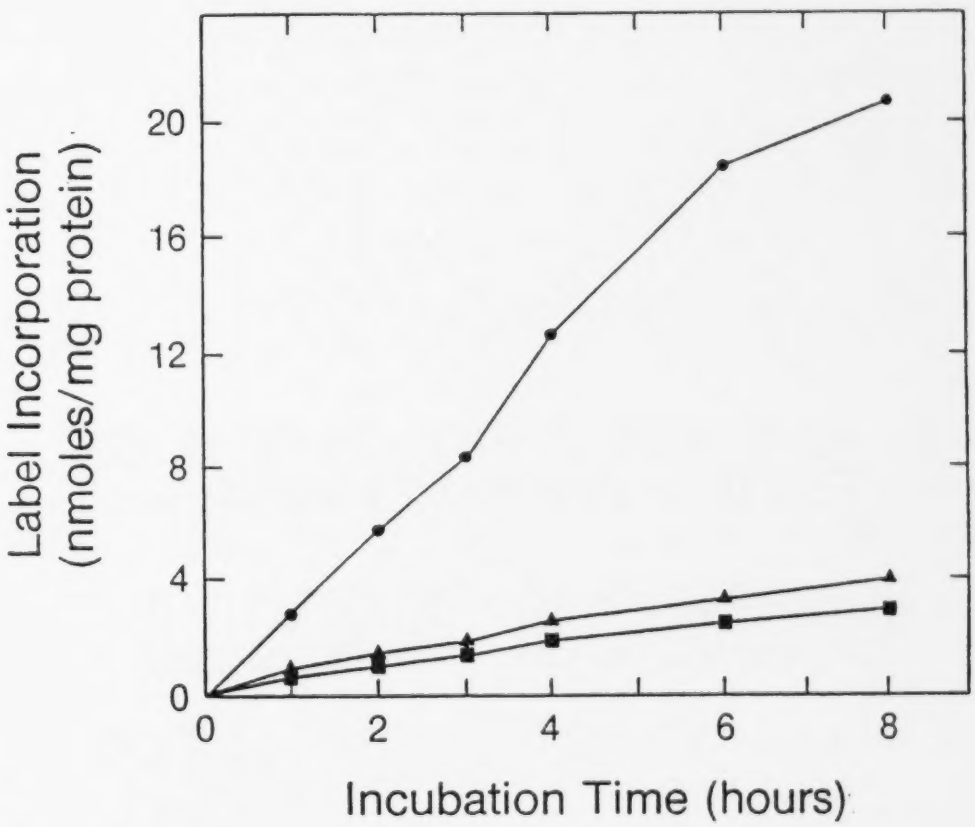

Figure IV.2 Time dependence of labelled fatty acid incorporation into the phospholipid fractions of the epithelial cell membrane.
The cultured cells were incubated with 1 μCi (100 nmoles) of [1-^{14}C]-18:2(6) for various times as indicated (at 37°C). Lipids were extracted, separated on TLC plates and counted with a scintillation counter. Values are the mean of 2 the experiments. (Variation < 10%).
(● - ●), phosphatidylcholine; (▲ - ▲), phosphatidylinositol; (■ - ■), phosphatidylethanolamine.

Separation of the total phospholipid into specific phospholipid fractions showed that phosphatidylcholine (PC) contained most of the radioactivity and that the pattern of label incorporation into individual phospholipids was different over 8 h incubation (Figure IV.2). The radioactivity in phosphatidylcholine increased rapidly while that in phosphatidylinositol (PI) and phosphatidylethanolamine (PE) increased slowly. As a result, an alteration in distribution of labelled fatty acid among

Table IV.1
Distribution of radioactivity among phospholipid fractions after various incubation times

Time Lapse (h)	Distribution of Radioactivity (%)[a] 1	2	3	4	6	8
PC[b]	65.5 (54-67)	71.5 (71-72)	72.0 (71-73)	73.5 (73-74)	76.5 (76-77)	76.5 (76-77)
PI	19.5 (19-20)	16.0 (16-16)	15.5 (15-16)	15.0 (15-15)	13.0 (13-13)	13.0 (13-13)
PE	15.0 (14-16)	12.5 (12-13)	12.5 (12-13)	11.5 (11-12)	10.5 (10-11)	10.5 (10-11)

Data was calculated from Figure IV-2. Values are the mean of 2 experiments.
Extreme values between brackets.
a, values are expressed as percent of labelled fatty acid (in nmoles)in each phospholipid and divided by the sum of the nmoless of labelled fatty acid in the three phospholipids.
b, PC=phosphatidylcholine, PI=phosphatidylinositol, PE=phosphatidylethanolamine.
PS did not contain significantly radioactivity.

phospholipid fractions (expressed as percentage) was seen during the incubation period (Table IV.1). Overall, the percentage of radioactivity in phosphatidylcholine increased in the 8th h compared with the 1st h, from 65.5% to 76.5%, while the percentage of radioactivity in phosphatidylinositol and phosphatidylethanolamine decreased from 19.5% to 13% and from 15% to 10.5%, respectively.

To compare 18:2ω6 incorporation with non-essential fatty acid incorporation in the cells, [1-C^{14}]-18:1ω9 and [1-^{14}C]-16:0 were incubated with the cells under

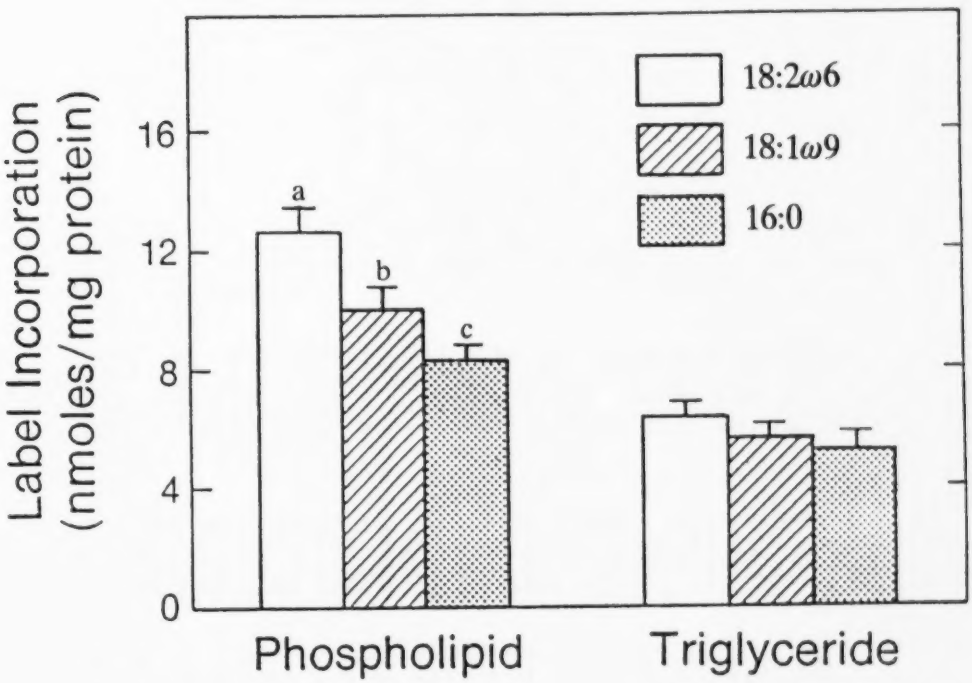

Figure IV.3 Comparison of 18:2ω6, 18:1ω9 and 16:0 incorporation into phospholipid and triglyceride of the cultured cells.
The same concentration (1 μCi, 100 nmoles)of [1-^{14}C]-18:1ω9 and [1-^{14}C]-16:0 was incubated with the cells under identical conditions for 4 h. Lipids were extracted, separated on TLC plates and counted with a scintillation counter. Values are the means ± SD of three experiments.
a,b,c, difference between groups, $P<0.05$

identical conditions as ^{14}C-18:2ω6. The amount of fatty acid incorporated into phospholipid was in the order: 18:2ω6 > 18:1ω9 > 16:0, while the amount of these fatty acids incorporated into triglyceride was not different (Figure VI.3), suggesting that these cultured cells preferentially incorporate polyunsaturated fatty acid into the phospholipid fraction.

To test the effect of other polyunsaturated fatty acid supplementation on 18:2ω6 incorporation in the cells, [1-^{14}C]-18:2ω6 plus 50 μM of 20:5ω3 or 20:4ω6 was

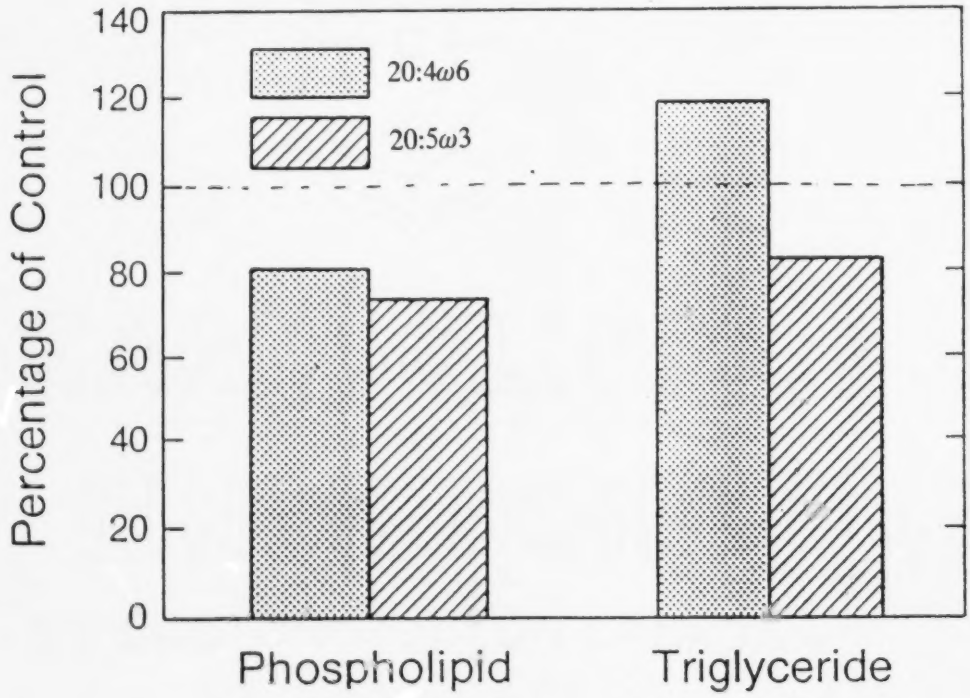

Figure IV.4 Effect of 20:4ω6 and 20:5ω3 supplementation on [1-^{14}C]-18:2ω6 incorporation into phospholipid and triglyceride of cultured epithelial cells.
50 µM unlabelled fatty acid (20:4ω6, 20:5ω3) was incubated for 4 h with 10 µM [1-^{14}]-18:2ω6. Values are means of two separate experiments in duplicate determinations (variation < 10%) and are compared to the control with (no competitor fatty acid present, expressed as 100%,-----)

incubated with the cells. After 4 h incubation with 20:5ω3, [1-^{14}C]-18:2ω6 incorporation into phospholipid and triglyceride was reduced by 28% and 16% respectively, while 20:4ω6 supplementation reduced [1-^{14}C]18:2ω6 incoporation into phospholipid by 15% but increased incorporation into triglyceride by 20% (Figure IV.4). These results are similar to those found in cultured human fibroblasts (Takahushi et al., 1991).

Desaturation and elongation of labelled fatty acid

The conversion of [1-^{14}C] fatty acid to its desaturation and elogation products

Table IV.2 Conversion of [1-^{14}C]18:2ω6 to its various desaturation and elongation products by cultured human airway epithelial cells

Fatty acids	Radioactivity distribution (%)
Diene	70±1.2
Trienes 18:3ω6	7.1±1.4
20:3ω6	12.7±3.0
Tetraenes	9.0±2.6
Pentaenes	1.1±0.6

Cells grown for 5-7 days with MEM/F_{12} + Hormone + 1% serum were incubated with [1-^{14}C]-18:2(6) (1 μCi, 100 nmoles) for 4 h in a similar medium without serum.
Lipids were extracted, methylated and separated on $AgNO_3$-TLC plates. The labelled fatty acids in silica were scraped and counted with a scintillation counter. Results are the mean of 11 cultures ± SD.

was determined by detection of radioactivity in fatty acid methyl esters. The conversion was compared to control samples in which cells were heated in boiling water for 5 min before addition of the same amount of labelled substrate fatty acid. It is apparent that human airway epithelial cells are capable of chain elongating-desaturating [1-^{14}C]-18:2ω6 to synthesize homologous trienes, tetraenes and pentaenes (Table IV.2), indicating that the cells in culture contain active Δ^6- and Δ^5-desaturase activity. Products compatible with Δ^4-desaturase activity or an alternate pathway (Voss et al., 1991) were also observed. After 4 h incubation, approximately 8.2

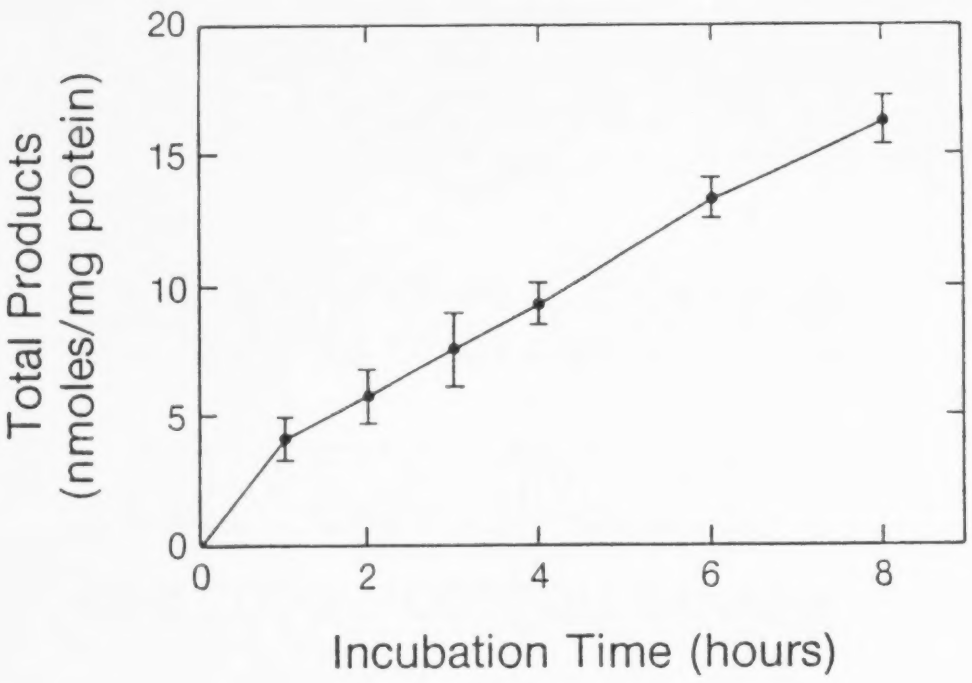

Figure IV.5 Time-dependent increase of products formed from [1-^{14}C]-18:2ω6 in cultured epithelial cells.
Cells were incubated with 1 μCi (100 nmoles) of [1-14C]-18:2ω6 for various times as indicated. Lipids were extracted, methylated and separated by TLC. Values are the means ± SD of three experiments.

nmoles of total products/mg cellular protein, accounting for 20-30% of fatty acid incorporated, was formed form 18:2ω6. The major products were 20:3ω6 and 20:4ω6, with less 18:3(6), suggesting elongation from 18:3ω6 to 20:3ω6 occurs very quickly (Table IV.2). This finding is similar that found in cultured glioma cells (Cook, 1987). The ratio of trienes to tetraenes was about 2:1. A time-dependent increase in total desaturation products was observed during 8 h incubation, suggesting active desaturase activities (Figure IV.5).

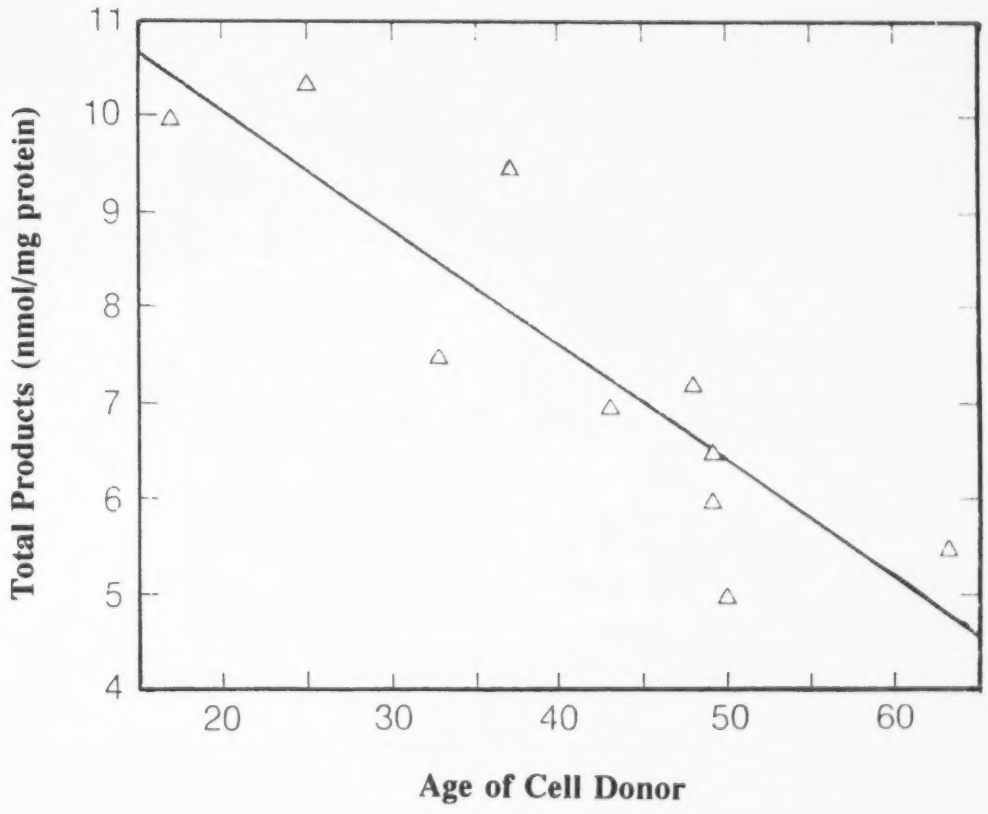

Figure IV.6 Relationship between desaturase activity and cell donor's age.
Details of desaturase assay as described in Table IV-2. Regression analysis: r=0.89.

A linear relationship was apparent between cell donor's age and desaturase activity observed (Figure IV.6, r=0.89). The total desaturation products of 18:2ω6 decreased as the donor's age advanced, indicating that the desaturase activity is age-related. This result is consistent with previous findings in other cell types (Takahashi, 1991; Choi et al., 1988; Bordone, 1988; Hrelia, 1989).

When compared to [1-^{14}C]-18:2ω6, the percent conversion rate of [1-^{14}C]-18:1ω9 was relatively lower (Table IV.3). When [1-^{14}C]-16:0 was provided to the cells, 23% of [1-^{14}C]-16:0 incorporated was converted to higher homogenous fatty acids, the major product was 16:1, indicating that a high level of Δ^9-desaturase activity occurs in epithelial cells (Table IV.3).

Table IV.3
Conversion of [1-^{14}C]-18:1ω9 and 16:0 to various desaturation and elongation products by cultured human airway epithelial cells.

Substrate provided	[1-^{14}C]-16:0	[1-^{14}C]-18:1 ω9
Fatty acid	Distribution of radioactivity (%)	
Saturated	73	
Monoenes	13.3	82
Dienes	10.5	7.6
Trienes	2.8	10.4

Cells were incubated as described for Figure IV-3. Fatty acids were methylated and separated on TLC plates. Results are the means of two experiments (variation <10%).

When supplemented to culture medium, 20:4ω6 and 20:5ω3 reduced conversion of [1-^{14}C]18:2ω6 to its products by 50% and 70%, respectively (Figure IV.7). This effect of 20:5ω3 on desaturation of 18:2ω6 has been suggested in previous studies using microsomes isolated from rats fed a fish-oil enriched diet (Garg and Clandinin, 1988; Kurata, 1980) and using cultured human fibroblasts (Takahashi, 1991). 20:4ω6 inhibition of 18:2ω6 conversion has been reported in other cell types (Takahashi, 1991; Cook, 1987; Ataniz, 1990).

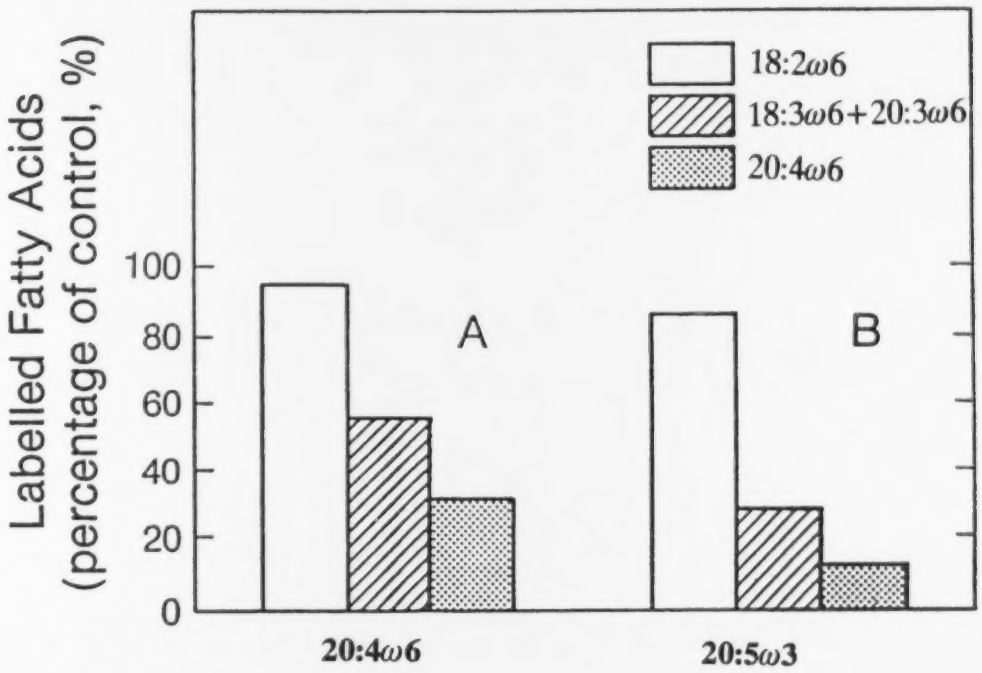

Figure IV.7 Influence of 20:4ω6 and 20:5ω3 on desaturation of [1-^{14}C]-18:2ω6
Cells were incubated as described for Figure IV-4 and lipid extracts were methylated, separated on $AgNO_3$-TLC plates and counted with a scintillation counter. Values are the means of two experiments in duplicate determinations (variation <10%) and are compared to the control (without competitor fatty acid, expressed as 100%). A: 50 μM unlabelled 20:4ω6 added. B: 50 μM unlabelled 20:5ω3.

Distribution of labelled fatty acyl chains in individual lipid fractions

The distribution of [1-^{14}C]-18:2ω6 and its products among phospholipid fractions and triglyceride after incubation of 100 nmole [1-^{14}C]-18:2ω6 with $5x10^5$ cells for 4 h is shown (Table IV.4). The profile of labelled fatty acids in individual lipid fractions was different. In phosphatidylcholine and triglyceride, the major labelled fatty acid was 18:2ω6, whereas phosphatidylinositol and phosphatidylethanolamine have a relatively higher proportion of 20:4ω6 and 20:3ω6, suggesting that the higher unsaturated fatty acids are preferentially incorporated into phosphatidylinositol and phosphatidylethanolamine.

Table IV.4
Distribution of labelled fatty acid chains in individual lipid fractions of cultured epithelial cells

	C18:2ω6	Trienes	Tetraenes
	Radioactivity (%)		
Phosphatidylcholine	85.0±3.1	11.7±2.3	2.6±0.5
Phosphatidylethanolamine	59.1±7.7	25.0±3.8	15.7±4.0
Phosphatidylinositol	52.0±7.8	31.5±6.4	16.5±1.5
Triglyceride	82.4±7.5	14.0±5.2	3.6±2.0

Cells were incubated with [1-^{14}C]-18:2ω6 for 4 h. Lipids were extracted, separated on silica gel-H plates to individual lipid fractions. Fatty acid chains of lipid fractions were methylated, separated on $AgNO_3$-TLC plates and counted with a scintillation counter. Values are the means of 5 experiments ± SD.

Release of products formed from [1-^{14}C]-18:2ω6

Released fatty acids formed from [1-^{14}C]-18:2ω6 were determined by quantitation of labelled trienoic and tetraenoic fatty acids (18:3, 20:3, 20:4) extracted from culture medium following 4 h incubation. The amount of the fatty acids released to culture medium (except 18:2ω6) was about 1.2 nmoles/mg protein of cells, accounting for 10-15% of total products formed. The major fatty acid was 18:3ω6 (60%), while 20:3ω6 and 20:4ω6 accounted for 25% and 15%, respectively. Release of fatty acid was not due to cell death or injury since cell viability was found to be 98-100% by trypan blue exclusion after incubation.

Turnover rate of phospholipid fatty acid chain

Turnover of phospholipid fatty acyl chains in cultured airway epithelial cells was determined by a pulse-chase experiment in which a 2 h pulse of [1-^{14}C]-18:2ω6 was followed by chase periods of 1-4 h. Following a 4 h chase, the radioactivity in the major phospholipids (PC, PE and PI), particularly in phosphatidylcholine, significantly decreased, while the fatty acid label in phosphatidylserine and triglyceride slightly increased (Figure IV.8). The label decrease in PC, PI and PE during the first two hours of chase was faster than that during the last two hours (2-4 h). Accordingly, fatty acid label released to the medium increased with the decrease in phospholipids (Figure IV.8) The half-life of fatty acid chain in PC, PI and PE was estimated to be approximately 5.5, 6 and 7.4 h, respectively.

Analysis of lipid mass (phosphorus content) and label distribution in individual phospholipids showed that PC, PI, PE and PS comprise 53.2%, 12%, 28.3% and 6.5% of the total lipid mass, respectively, while labelled fatty acids found in these phospholipids were 73%, 13%, 12% and 2% of total labelled fatty acids incroporated, respectively (Table IV.5). These data also suggest that the order of turnover rate of phospholipid fatty acid chain is PC > PI > PE > PS.

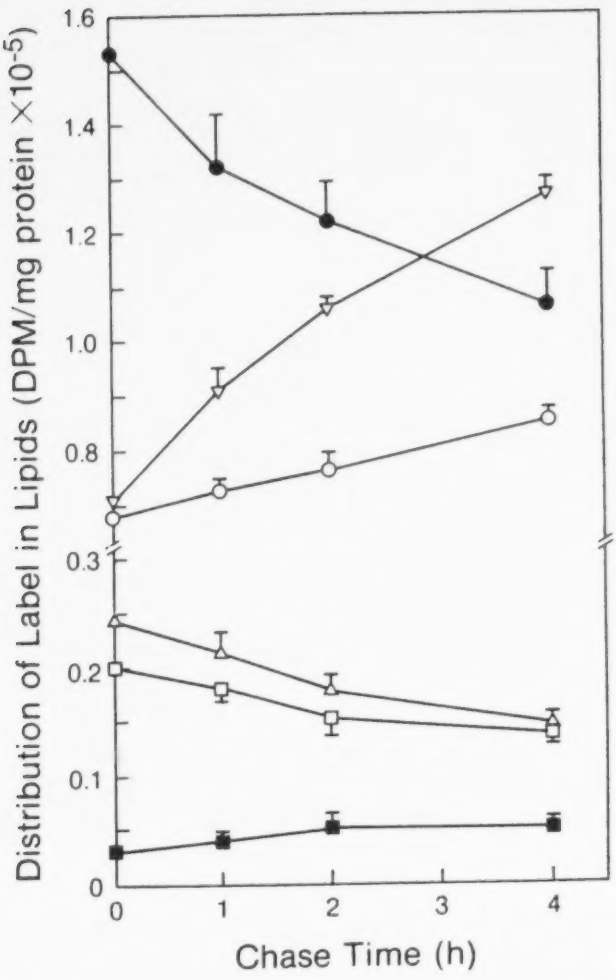

Figure IV.8 Distribution of radiolabel [1-^{14}C]-18:2ω6 in the lipid fractions of cultured airway epithelial cells during chase with 18:2ω6.

Cells were pulse-labelled with [1-^{14}C]-18:2ω6 (1 μCi/dish) for 2 h. Radioactive medium was removed and cells were washed once with o.5% bovine serum albumin in phosphate-buffered saline. Fresh medium containing 100 μM unlabelled 18:2ω6 for various time. After the chase, harvested cells were washed once. Lipids were extracted and separated into neutral and phospholipid classes by TLC. Results are expressed as total dpm in lipid fractions/mg protein. ●-●, phosphatidylcholine; Δ-Δ, phosphatidylinositol; □-□, phosphatidylethanolamine; ■-■, phosphatidylserine; ○-○, triglyceride; ▽–▽, culture medium. values are mean of observations of cell cultured from four individuals.

Table IV.5 Comparion between quantity and label incorporation of individual phospholipids

Phospholipids	(mol %)[a]	Distribution of label (%)[b]
Phosphatidylcholine	53.7±3.1	73±3
Phosphatidylethanolamine	28.3±1.5	12±1.8
Phosphatidylinositol	11.5±1.0	13±2.3
Phosphatidylserine	6.5±1.0	2.0±0.5

[a]Lipid extracts of cultured human airway epithelial cells were fractionated using TLC and then assayed for phosphorus content. Values are expresed as percent of phosphorus (in moles) in each phospholipid and divided by the sum of the phosphorus in the four phospholipids. Results are the means of three experiments.

[b]Cultured human airway epithelial cells were incubated with [1-^{14}C]-18:2(6) (2.2 x 10^6 dpm/100nmoles) for 4 h. Lipids were extracted, separated on TLC and counted with a scintillation counter. Values are expressed as percent of radiolabel in each phospholipid class and divided by the sum of the radiolabel in the four phospholipids. Results are the means of 5 experiments.

Effect of fatty acid supplementation on fatty acid composition

To examine the effect of fatty acid supplementation to culture medium on fatty acid composition of phospholipid, the fatty acid profile of total phospholipids from cell incubated with or without 100 μM 18:2ω6 was analyzed (Table IV.6). It is apparent that after fatty acid supplementation the levels of both 18:2ω6 and 20:4ω6 increased significantly and are close to those found in freshly isolated cells (Alpert,1991).

Table IV.6 Fatty acid profile of phospholipids of cultured human airway epithelial cells with and without 18:2ω6 supplemenation

Fatty acid [a]	Composition (% w/w)	
	None [b] ($n = 4$)	Linoleic acid (18:2ω6) [c] ($n = 3$)
16:0	15.8 ±2.6	16.1 ± 1.2
16:1	4.5 ±1.2	6.1 ± 0.3
18:0	13.4 ±3.3	11.3 ± 0.2
18:1ω9	33.0 ±5.1	23.5 ± 2.0 *
18:1ω7	11.0 ±2.1	10.1 ± 0.9
18:2ω6	2.6 ±0.2	10.9 ± 0.7 *
18:3ω3	0.57 ± 0.1	0.3 ± 0.1
20:1ω9	0.74 ± 0.1	–
20:3ω9	1.2 ±0.43	0.2 ± 0.1 *
20:3ω6	2.6 ±0.4	4.2 ± 1.5 *
20:4ω6	4.3 ±1.0	6.1 ± 0.3 *
20:5ω3	1.4 ±0.3	0.2 ± 0.1
22:4ω6	0.5 ±0.2	1.3 ± 0.2 *
22:5ω6	0.25 ± 0.1	0.6 ± 0.15
22:5ω3	1.1 ±0.5	1.0 ± 0.2
22:6ω3	1.7 ±0.65	1.9 ± 0.6
ΣSFA	31.3 ±3.5	29.4 ± 2.1
ΣMUFA	54.2 ±5.9	43.9 ± 3.5 *
Σω6	10.2 ±2.7	23.5 ± 1.2 *
Σω3	3.6 ±1.4	3.3 ± 0.5

[a] Fatty acids analyzed included all saturated and unsaturated fatty acids of 16 to 22 carbons in chain length.

[b] Growth medium containing no supplemental fatty acid.

[c] Growth medium supplemented with 18:2ω6 (100 μM). The cells were exposed to the 18:2ω6 for 4 h.

ΣSFA = total saturated fatty acids; ΣMUFA = total monounsaturated fatty acids; Σω6 = total ω6 fatty acids; Σω3 = total ω3 fatty acids.

values are means ± S.D., * $P < 0.05$.

n = cell cultures from different subjects.

Time -dependent changes in distribution of labelled fatty acids in the major phospholipids during the period of pulse and chase was also examined. This data is shown in Appendiix I.1. Fatty acid composition of individual phospholipids from the cultured epithelial cells was also analyzed and is presented in Appendix I.2.

Discussion

Although fatty acid metabolism of cultured mammalian cells has been extensively investigated (reviewed by Spector, 1981; Rosenthal, 1987; Brenner, 1974) and the capability to incorporate and desaturate exogenous fatty acids has been demonstrated in many animal tissues (Garg, 1988; Clandinin, 1985; Tocher, 1990) as well as culture cell lines (Maeda et al., 1978; Tadahashi, 1991; Pautline, 1986; Whatley, 1990; Cook, 1983; Alaniz, 1975), there is little information characterizing esential fatty acid metabolism in human epithelial cells. This study has clearly demonstrated that human airway epithelial cells in primary cultures are able to rapidly incorporate exogenous fatty acyl chains into cellular lipids and actively desaturate and elongate them to higher polyunsaturated fatty acids; and fatty acyl chains of the epithelial phospholipid are in a dynamic state of rapid turnover.

From the patterns of change in incorporation of the [1-^{14}C]-18:2ω6 over time in the cell at least two distinctive phases for metabolism of label were identified. The first phase, during the initial 2 h after addition, is characterized by a rapid labelling of the neutral lipid followed by slower labelling of phospholipids. The second phase, from 3-8 h, is characterized by a very slow increase (almost constant) in labelling of triglyceride and a continuing increase in labelling of phospholipids, particularly phosphatidylcholine fraction. These results are similar to those in previous studies using different cell types and labelled fatty acids (Dhopeshwarker et al., 1973; Sun and Yan, 1976). The pattern of incorporation observed in the second phase may result from a larger fatty acyl chain pool of phospholipid or a shift of fatty acyl chains from triglyceride to phospholipid following longer incubation.

Analysis of distribution of radioactivity among individual phospholipids shows that the amount of 18:2ω6 incorporated into individual phospholipid classes in the cultured cells was in the order: phosphatidylcholine > phosphatidylinositol > phosphatidylethanolamine. This result is similar to that found previously in the same cell type incubated with [1-^{14}C]-20:4ω6 (Haltzman, 1988), but different from that found in cells of neural origin (Tocher, 1990) (phosphatidylcholine > phosphatidylethanolamine > phosphatidylinositol). The present study also demonstrates that the composition of labelled fatty acid of individual phospholipid classes is distinctive: phosphatidylinositol and to a lesser extent phosphatidylethanolamine have higher levels 20:4ω6 and 20:3ω6 compared to phosphatidylcholine and triglyceride which have a higher level of 18:2ω6. These results indicate that desaturation products of 18:2ω6 are preferentially incorporated into phosphatidylinositol and phosphatidylethanolamine.

It is evident that human airway epithelial cells possess active Δ9-, Δ6-, Δ5-desaturase and thereby are able to produce 20:4ω6 and other polyunsaturated fatty acids. Thus, the low level of 20:4ω6 in the cultured cells reported by Alpert et al. (1991) should not be the result of a lack of Δ6-desaturase, but may be due to an insufficiency in substrate (18:2ω6) for Δ^6-desaturase. This is supported not only by the results of the assay of [^{14}C]18:2ω6 conversion, but also by analysis of fatty acid composition of membrane phospholipid, which showed that 18:2ω6 supplementation to culture medium increased both 18:2ω6 and 20:4ω6 to levels close to those found in freshly isolated cells. It is notable that desaturation rate is age-related. Therefore, age could be an important physiological factor affecting desaturase activity, thereby affecting fatty acid composition of cell membrane. The presence of desaturase enzymes in the airway epithelium suggest that: 1) the cells have capability to produce a variety of polyunsaturated fatty acids which can be esterified to phospholipids,

thereby altering the composition of phospholipid fatty acyl chains; 2) pathologically induced alterations in both membrane fatty acid composition and eicosanoid production in pulmonary diseases may, in part, be mediated by changes in desaturase activities, thereby altering the type of fatty acids available for cell membrane synthesis and resulting in a low level of a specific polyunsaturated fatty acid in cell membrane.

Our results clearly show that 20:5ω3 (50 μm) inhibited incorporation of radioactive linoleic acid (18:2ω6) and reduced radioactivity in metabolites formed by desaturation-elongation. This effect of 20:5ω3 may indirectly reduce arachidonic acid (20:4ω6) availability for synthesis of cellular structure and prostaglandins, by competing with the incorporation of 18:2ω6 and 20:4ω6, and inhibiting the pathway for biosynthesis of 18:3ω6 and 20:4ω6.

Arachidonic acid (20:4ω6) inhibited 18:2ω6 incorporation only into phospholipid, and increased 18:2ω6 incorporation into triglyceride. Exogenous 20:4ω6 may compete with 18:2ω6 for incorporation into cellular phospholipid, and thus influence both transfer of 18:2ω6 between the major lipid pools and the activity of the desaturation-elongation systems in the epithelial cells. However, in neuroblastoma cells, 20:4ω6 apparently stimulates the desaturation of [1-^{14}C]-18:2ω6 (Cook, 1983). It is possible that the intracellular interactions between fatty acids are not universal in all cell types.

Our observation that the higher unsaturated fatty acids were incorporated more into cellular lipids supports the hypothesis that polyunsaturated fatty acids have a higher affinity for acyl-CoA synthetase and/or acyltransferase (Rosenthal, 1987).

During the chase after a 2 h pulse, with a decrease in the radioactivity of phospholipids, the radioactivity in the medium increased progressively, suggesting that the cellular esterified fatty acid was released to the medium when free fatty acids were added. This finding is in agreement with the earlier studies using other cell

types (Chakravarthy, 1986; Spector and Steinberg, 1967; Tasi, 1978). The increase in triglyceride radiolabel during the chase may be due to an enhanced triglyceride synthesis which may accommodate an influx of fatty acid or act as a trap of released acyl chain from phospholipids when 18:2ω6 was added to the cultured medium, as suggested by earlier studies (Chakravarthy, 1986; Tsai, 1978).

On the basis of previous conceptions and the findings in this study, a systematic pathway for 18:2ω6 metabolism in human airway epithelial cells is proposed as illustrated in Appendix I.3.

Chapter V

THE CHLORIDE CHANNEL BLOCKER ANTHRACENE-9-CARBOXYLATE INHIBITS FATTY ACID INCORPORATION INTO PHOSPHOLIPID IN CULTURED HUMAN AIRWAY EPITHELIAL CELLS

Introduction

Cystic fibrosis is a lethal inherited disease with a high incidence in the Caucasian population. The primary defect of the disease is the failure of an epithelial cell chloride channel to respond to cAMP (Frizzell et al.,1986; Welsh, 1986; Li et al., 1988; Quinton, 1989). As a result, the permeability of epithelial tissues to Cl^- is lost or severely reduced. In airway cells this leads to an imbalance in ion and fluid transport, causing abnormal mucus secretion and ultimately resulting in pulmonary infection and epithelial cell damage. One of the principal features of the disease also involves low essential fatty acid levels (particularly 18:2ω6), independent of pancreatic insufficiency and therefore not the result of malnutrition (Galabert, 1978; Rogiers, 1980; Rogiers, 1983; Chase, 1978). Although the possibility of an increased turnover of essential fatty acids has been proposed (Rogiers, 1982; Chase, 1980; Roscher, 1981; Gilljam, 1986; Strandvik, 1989), definition of the mechanism of the abnormality remain controversial. It is also uncertain that the reduced membrane level of 18:2ω6 is related to the pathophysiology of the disease. Whether or not impermeability of the epithelial cell membrane to Cl^- and other ions affects fatty acid metabolism is also unknown. Therefore the present study was initiated to examines the effect of anthracene 9-carboxylic acid (9-AC), under conditions known to inhibit chloride conductance across epithelial membrane (Welsh, 1984; Wong, 1988; Welsh, 1986; Gogelein, 1988), on incorporation and desaturation of essential fatty acid,

18:2ω6 in cultured human airway epithelial cells.

Methods

The methods used in this study include cell isolation, primary cell culture, isotope incubation, lipid extraction, thin layer chromatography, fatty acid analysis, liquid scintillation counting and protein determination and are described in detail in Chapter III.

Results

Effect of 9-AC on [1-^{14}C]18:2ω6 incorporation

To determine if 9-AC alters [1-^{14}C]18:2ω6 incorporation into cellular lipids, 5 mM 9-AC dissolved in dimethyl sulfoxide (DMSO) was added to the culture medium. After 4 h of incubation, lipid analysis revealed that label incorporation into phospholipid was reduced by 60%-70% (17.9 ± 3.5 to 5.5 ± 2.1 nmol/mg of protein, n = 5), whereas label incorporation into triglyceride increased by 50%-100% (7.5 ±2.8 to 12.3 ± 4.0 nmol/mg of protein, n = 5) (Figure V.1). To determine if DMSO produced effects similar to those observed with 9-AC, DMSO alone was added and found not alter 18:2ω6 incorporation. Quantification of total phospholipid by phosphorus determination indicated no difference in phospholipid mass between control and 9-AC-treated cultures, suggesting that low levels of 18:2ω6 observed after treatment of cells with 9-AC are not due to loss of phospholipid, but result from inhibition of 18:2ω6 incorporation into phospholipid. Separation of total phospholipid showed that the inhibition of 18:2ω6 incorporation into phosphatidylcholine, phosphatidylethanolamine and phosphatidylinostol by 5 mM 9-AC was approx. 70%, 62% and 60% respectively.

The inhibition of 18:2ω6 incorporation into phospholipid by 9-AC was rapid

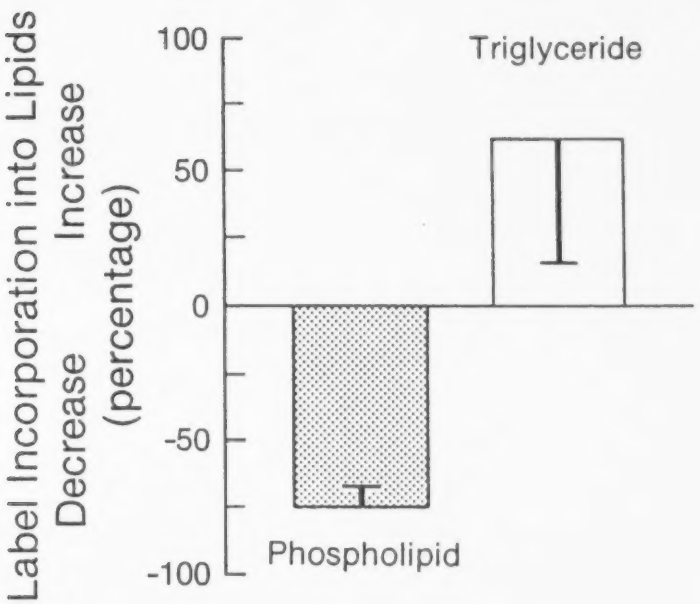

Figure V.1 Effect of anthracene -9-carboxylate on [1-^{14}C]18:2ω6 incorporation into phospholipid and triglyceride of cultured human airway epithelial cells
Anthracene-9-carboxylate (5mM) was added to the culture medium. After 5 min, 100 n mole (1μCi) of [1-^{14}C]18:2ω6 was added and incubated for 4 h. Cellular lipids were extracted and separated on TLC. Radioactivity in phospholipid and triglyceride was counted by liquid scintillation counting. Results were compared with control in percentage. Values are the means ± SD of 5 experiments. Incorparation of label into CE was low.

(Figure V.2) and concentration-dependent (Figure V.3). The inhibition had reached a stable state by approx. 30 min; 5 mM 9-AC decreased 18:2ω6 incorporation into phospholipid by 60%. The effect of another Cl^- channel blocker, diphenylamine 2-carboxylate (DPC), on 18:2ω6 incorporation has also been tested. The result indicated that 2 mM DPC decreased 18:2ω6 incorporation into phospholipid by appox. 60% (17.2 ± 0.2 to 7.0 ± 0.2 nmol/mg protein, n = 3) and increased 18:2ω6 incorporation into triglyceride by 64% (6.5 ± 0.7 to 11.0 ± 0.6 nmol/mg of protein, n = 3).

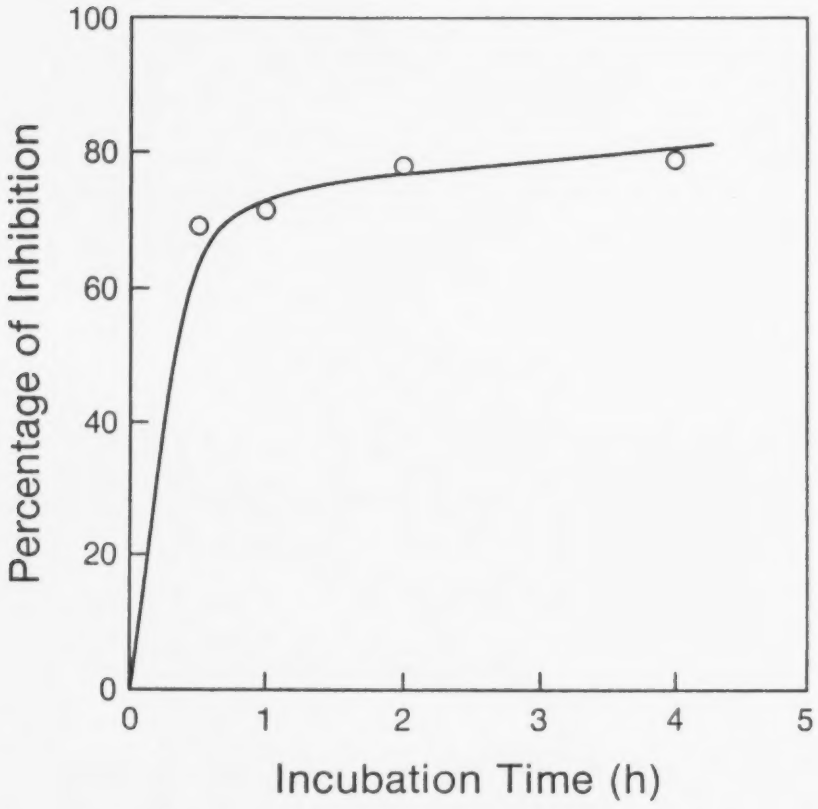

Figure V.2 Time course of the effect of anthracene-9-carboxylate on [1-^{14}C]-18:2ω6 incorporation into phospholipid

Culture epithelial cells were incubated with [1-^{14}C]-18:2ω6 (1 μCi) with or without anthracene-9-carboxylate (5mM) for various times as indicated. Lipids were extracted and separated on TLC. Radioactivity of phospholipids was counted by a scintillation counter. Results were expressed as percent inhibition calculated from counting (DPM) in control and anthracene-9-carboxylate treated samples. Values are the means of two experiments in duplicate.

Cl^- specificity of the effect of 9-AC

To examine the Cl^- specificity of the inhibition of 18:2ω6 incorporation into phospholipid by 9-AC, epithelial cells were incubated with NaCl buffer solution and Cl^--free buffer solution (gluconate substitution). Radiolabel incorporation into

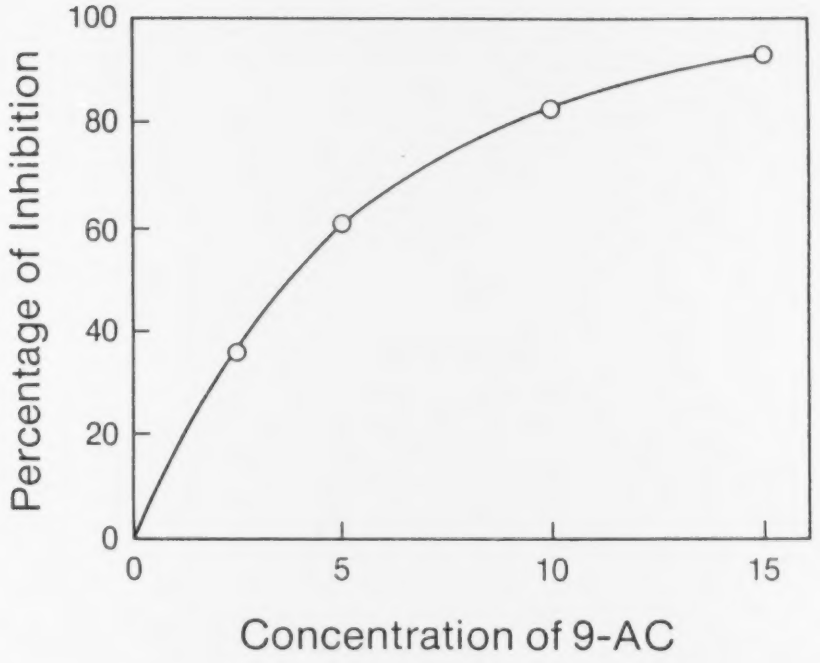

Figure V.3 Effect of anthracene-9-carboxylate concentration on [1-^{14}C]-18:2ω6 incorporation into phospholipid

Anthracene-9-carboxylate was added to the culture medium to produce final concentrations as indicated. Cells were incubated with [1-^{14}C]-18:2ω6 for 4 h. Lipids were extracted and separated on TLC. Radioactivity of phospholipid was counted by a scintillation counter. Results were expressed as percent inhibition compared with the control. Values are the means of two experiments in duplicate.

phospholipid in Cl^--free buffer solution was much lower than that in NaCl buffer solution and that the inhibition of 18:ω6 incorporation by 9-AC was much more in NaCl buffer solution than in Cl^--free buffer solution (Figure V.4). These results suggest that the effect of 9-AC may be via an inhibition of Cl^- conductance.

Furthermore, to determine whether other ion channel blockers produced effects similar to those observed with 9-AC, the effects of amiloride (a Na^+-channel

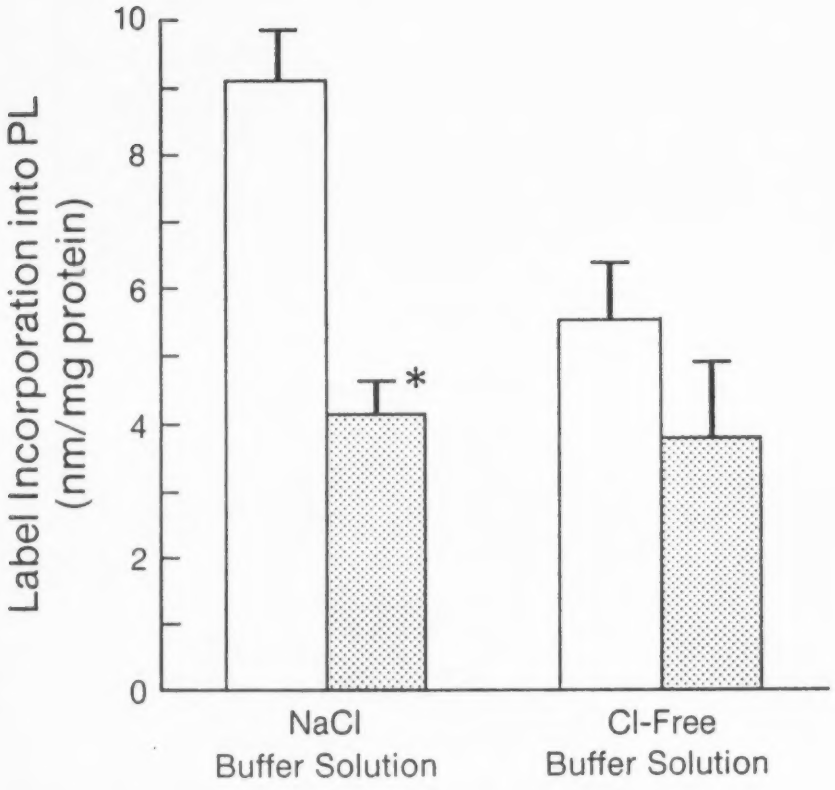

Figure V.4 Effect of ion substitution on the response to anthracene-9-carboxylate
The cultured epithelial cells were in cubated with [1-^{14}C]-18:2ω6 in either NaCl buffer solution (mM): 1.2 $CaCl_2$, 118.9 NaCl, 20.4 $NaHCO_3$, 2.4 K_2HPO_4 and 0.6 KH_2PO_4 or Cl^--free buffer solution (mM): 1.2 $CaSO_4$, 1.2 $MgSO_4$, 118.9 Na-gluconate, 20.4 $NaHCO_3$, 2.4 K_2HPO_4 and 0.6 KH_2PO_4, containing anthracene-9-carboxylate ($5x10^{-3}$M) ● or containing no anthracene-9-carboxylate (□) for 2 h. Lipids were extracted and separated on TLC. Radioactivity of phospholipid was counted by scintillation counter. Results are the means of three determinations, *$p<0.01$.

blocker) and verapamil (a Ca^{++}-channel blocker) on [1-^{14}C]18:2ω6 incorporation were tested under identical conditions to those used for 9-AC. There were no differences in radiolabel incorporation into cellular lipids between cells incubated with those

other ion blockers and controls (Figure V.5). These results further suggest that the effect of 9-AC on 18:2ω6 incorporation into cellular lipids is specific for Cl^- permeability.

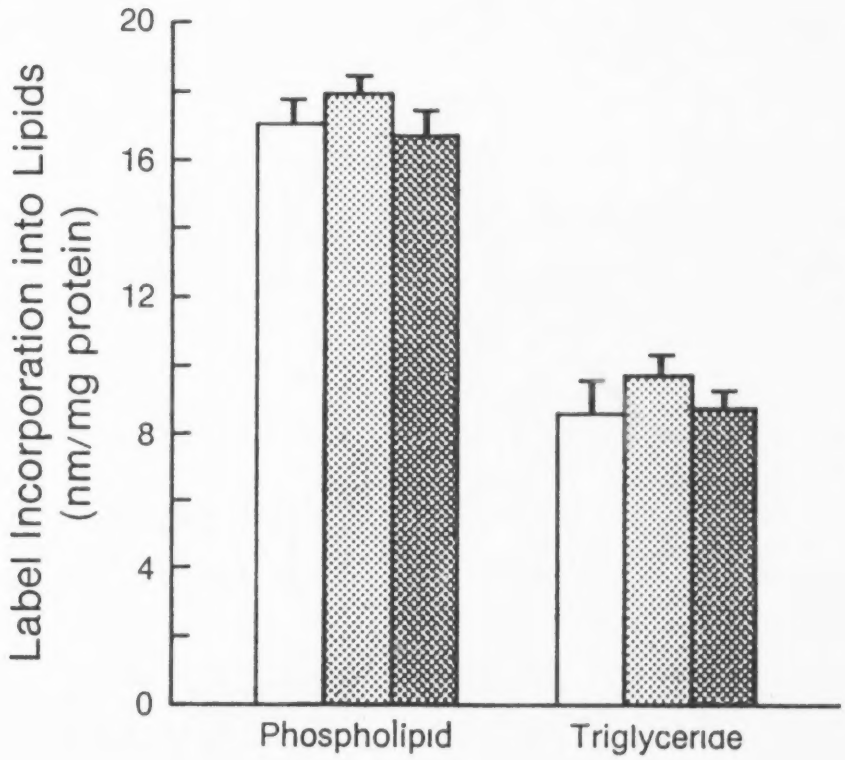

Figure V.5 Effect of ion (Na^+, Ca^{++}) channel blockers on [1-^{14}C]-18:2ω6 incorporation into cellular lipids

The cultured epithelial cells were incubated with [1-^{14}C]-18:2ω6 (1 μCi) in the medium containing verapamil ($2x10^{-5}$M) (▩), or amiloride ($2x10^{-5}$M) (▨) or no ion-channel blocker (control) (□) for 4 h. Lipids were extracted, separated on TLC and counted by scintillation counter. Results are the means of three experiments.

Effect of 9-AC on non-essential fatty acid incorporation

To test for a difference between the effects of 9-AC on essential fatty acid versus non-essential fatty acids, incorporation of [1-^{14}C]-18:1ω9, [1-^{14}C]16:0 and [1-^{14}C]18:2ω6 into cellular lipids was determined in the cells incubated with or without 9-AC. The decreases in [1-^{14}C]-18:2ω6, [1-^{14}C]18:1ω9 and [1-^{14}C]-16:0 incorporation into phospholipid by 9-AC were 63%, 56% and 38%, respectively (Figure V.6A). The increases in [1-^{14}C]18:2ω6, [1-^{14}C]18:1ω9 and [1-^{14}C]16:0 incorporation into triglyceride resulting from 9-AC treatment were 56%, 100% and 142%, respectively (Figure V.6B). It appear that incorporation of 18:2ω6 into phospholipid was inhibited more by 9-AC than was the incorporation of 18:1ω9 or 16:0.

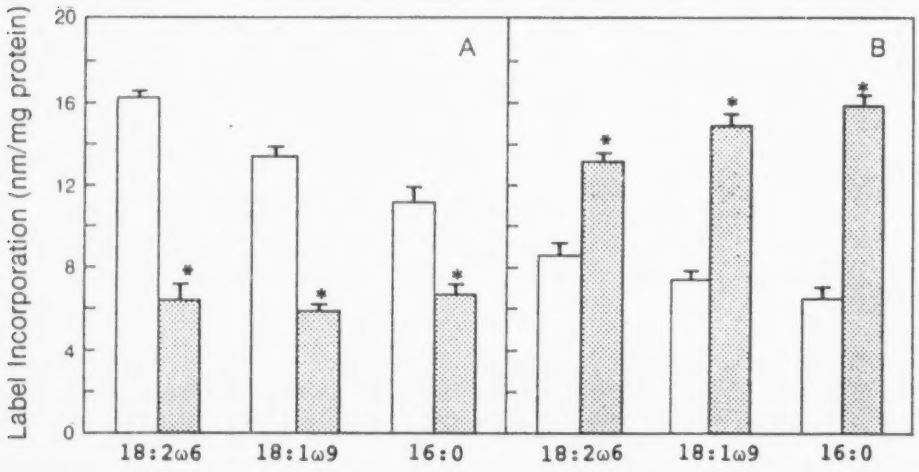

Figure V.6 Effect of anthracene-9-carboxylate on incorporation of fatty acids into cellular lipids (A: phospholipid, B: triglyceride)
Anthracene-9-carboxylate ($5x10^{-3}$M) was added to the medium. Cells were incubated with labelled fatty acids (1 μCi, 100 nmoles) for 4 h. Lipids were extracted, separated on TLC and counted by scintillation counter. Results are the means of three experiments. (▨) with anthracene-9-carboxylate; (□) without anthracene-9-carboxylate (*$P<0.05$).

Effect of 9-AC on fatty acid composition

Following a 4 h incubation with 50 μM 18:2ω6, with or without 10^{-2} M 9-AC, cellular lipids were extracted, and fatty acid composition was analyzed by gas-

Table V.1 Effect of 9-AC on fatty acid composition of phospholipids

Cells were incubated with 50 μM-18:2ω6 in medium containing no or 10 mM-9-AC for 4 h. Lipids were extracted, separated on t.l.c. and methylated with BF_3. Fatty acid methyl esters were separated and quantified by g.l.c. ΣSFA, total saturated fatty acids; ΣMUFA, total monounsaturated fatty acids; Σω6, total ω6 fatty acids; Σω3, total ω3 fatty acids. Values are means ± S.D. ($n = 3$). Significance of differences versus control: * $P < 0.05$; ** $P < 0.01$.

	Composition (%, w/w)	
Fatty acid	Control	9-AC
14:0	0.8 ± 0.3	0.5 ± 0.2
15:0	1.9 ± 0.2	2.1 ± 0.3
16:0	16.1 ± 1.2	17.3 ± 1.5
16:1	6.1 ± 0.3	5.7 ± 0.3
18:0	11.3 ± 0.2	12.0 ± 0.2*
18:1(ω9+ω7)	34.0 ± 3.5	37.0 ± 3.1*
18:2ω6	10.9 ± 0.7	5.5 ± 0.2**
18:3ω6	0.2 ± 0.1	–
18:3ω3	0.3 ± 0.1	0.3 ± 0.1
20:0	0.5 ± 0.2	0.6 ± 0.1
20:1ω9	–	0.7 ± 0.2*
20:3ω9	0.2 ± 0.1	0.3 ± 0.1
20:3ω6	4.2 ± 1.5	4.0 ± 1.8
20:4ω6	6.1 ± 0.3	6.2 ± 0.3
20:3ω3	0.2 ± 0.1	0.3 ± 0.1
22:5ω3	1.0 ± 0.2	0.9 ± 0.1
22:6ω3	1.9 ± 0.6	1.9 ± 0.5
ΣSFA	29.4 ± 2.1	31.5 ± 3.0
ΣMUFA	43.9 ± 3.5	47.3 ± 4.1
Σω6	23.5 ± 1.2	18.1 ± 1.1**
Σω3	3.3 ± 0.5	3.1 ± 0.5

chromatography. Cells incubated with 9-AC exhibited significantly lower level ($p<0.01$) of 18:2ω6, but higher levels of 18:1ω9 and 20:1ω9 (Table V.1). This pattern is similar to that found for cystic fibrosis in previous studies (Rivers, 1975; Rogiers, 1983; Chase, 1978).

Effect of 9-AC on 18:2ω6 desaturation

Desaturation of 18:2ω6 was determined in the cells incubated with and without 9-AC. The relatively conversion rate, based on the ratio of total products formed from 18:2ω6 to total substrate (18:2ω6) taken up by the cells, was not apparently changed by the addition of 9-AC (Table V.2).

Table V.2 Effect of anthracene-9-carboxylate on 18:2ω6 conversion

Fatty acid	Radioactivity distribution (%)	
	Control[a]	Anthracene-9-carboxylate[b]
18:2ω6	77 ± 7.0	78 ± 5.5
18:3ω6 & 20:3ω6	16 ± 3.5	14 ± 4.0
20:4ω6	7 ± 1.5	8 ± 2.5

Cells were incubated with [1-^{14}]18:2ω6 (1μCi, 100nM) for 4 h. Lipids were extracted, methylated and separated on $AgNO_3$ -TLC. Radioactivity was counted by liquid scintillation counting. Results are the means ± SD. of 4 experiments.

[a]Culture medium contained no anthracene-9-carboxylate.

[b]Culture medium contained 5 mM anthracene-9-carboxylate.

Discussion

The present study demonstrates that anthracene-9-carboxylic acid (9-AC) inhibits incorporation of fatty acids, particularly polyunsaturated fatty acids, into phospholipid of cultured human airway epithelial cells.

An effect of 9-AC on Cl^- conductance in epithelium has been well documented by previous studies (Welsh, 1984; Wong, 1988; Welsh 1986; Gogelein, 1988). 9-AC inhibited Cl^- secretion in airway epithelium by blocking an electrically conductive Cl^- exit step in the apical cell membrane. The inhibition was initially rapid, reaching a stable value by 10-20 min and was concentration dependent. 9-AC (4 mM) decreased the single-channel conductance of Cl^- channels in airway epithelium to 68% of control (Welsh, 1986), and 6 mM 9-AC inhibited Cl^- secretion by 50% in the intact epithelium (Welsh, 1984). Our observation that inhibition of fatty acid incorporation into phospholipid by 9-AC reached a stable state by approximately 30 min (Figure V.2); 5 mM 9-AC decreased 18:2 incorporation into phospholipid by 60% (Figure V.3) shows a pattern of effect of 9-AC on fatty acid incorporation similar to its known effects on Cl^- conductance, indicating a relationship between the blocking of Cl^- conductance across cell membrane and inhibition of fatty acid incorporation into membrane phospholipids. Further evidence for this relationship can be obtained from ion substitution studies (Figure VI.4). In the presence of Cl^-, 5 mM 9-AC inhibited 18:2ω6 incorporation into phospholipid by 60%. In the Cl^--free solution, total label incorporated into phospholipid was dramatically reduced and no significant effect by 9-AC was found. These results suggest that the effect of 9-AC on fatty acid incorporation involves Cl^- conductance or Cl- concentration.

Recently, a number of studies have demonstrated that polyunsaturated fatty acids, particularly arachidonic acid (20:4ω6), directly block Cl^--channel in airway epithelial cells, independent of change in membrane fluidity as well as enzymatic pathways (Hwang wt al., 1990; Anderson, 1990; Ordway, 1991). Our previous findings (Chapter IV) with airway epithelial cells illustrated that 20:4ω6 inhibited

18:2ω6 incorporation into phospholipid, but increased 18:2ω6 incorporation into triglyceride, analogous to the effect of 9-AC. Together, these results lead us to speculate that both 9-AC and 20:4ω6 may affect fatty acid incorporation into phospholipid at least in part by inhibition of Cl^- conductance.

Based on the finding that the total incorporation of labelled fatty acid into cellular lipids (phospholipids + neutral lipids) was decreased by 9-AC, the mechanism involved may be an effect on the transport of the fatty acid across the plasma membrane. However, the relationship between inhibition of Cl^- conductance and reduced fatty acid transport across the cell membrane is not known. We suggest three possibilities. First, the impermeability of the membrane to Cl^- results in changes in electrophysiological properties of the whole-cell thereby directly affecting the function of cellular protein. It is possible that most of the whole-cell currents are probably carried by chloride ions. Thus inhibition of Cl^- conductance could produce dramatic change in electrical potential across cell membrane (Welsh, 1986; Wong, 1988)). This may explain why Ca^{++}- and Na^+-channel blockers did not induce effects on fatty acid incorporation similar to those of 9-AC. Second, inhibition of Cl^- transport may change intracellular Cl^- concentration , altering intracellular pH and/or cellular volume, with subsequent effects on fatty acid incorporation. Cl^- channels have been shown to be involved in volume regulation in epithelial cells (Kolb, 1987), suggesting that cellular membrane can be mechanically modified (due to the volume change) by Cl^- conductance to influence the functions of membrane proteins. Third, both possibilities noted above may coexist. However, it may be that the effect of 9-AC is not due to its inhibition of Cl^- transport but caused by competition with fatty acid for incorporation into phospholipid. This appears less likely, because the precondition for 9-AC incorporation is formation of 9-AC-coA in which acyl-CoA synthase must have higher affinity to 9-AC than to 18:2ω6, and in this case 9-AC should inhibit 18:2ω6 incorporation into both phospholipid and triglyceride.

However, the high level of 18:2ω6 incorporation into triglyceride and the fatty acid profile observed did not support the possible competition of 9-AC with fatty acid.

It has been suggested that low level of 18:2ω6 found in membrane phospholipid of cystic fibrosis patients is caused by defective fatty acid metabolism (Rogiers, 1983; Chase, 1980; Gilljam, 1986; Strandvik, 1989). Although many studies have been concerned with the possibility of a defect in fatty acid metabolism in CF, little attention has been paid on mechanisms altering fatty acid incorporation. Active incorporation of fatty acids into the phospholipid of cellular membrane plays a major role in the continuous turnover of membrane lipids. Thus it is possible that a defect in incorporation of fatty acid could be partly responsible for the abnormal composition of epithelial membrane phospholipids observed in cystic fibrosis patients. In the present study, since 18:2ω6 incorporation into phospholipid was inhibited by the Cl^--channel blocker (9-AC), the effect of 9-AC was somewhat specific to essential fatty acid and the 9-AC induced change in fatty acid profile was characterized by a low level of 18:2ω6, it is thus logical to suggest that low levels of 18:2ω6 found in phospholipid in cystic fibrosis may be in part attributed to decreased incorporation of fatty acid resulting from the defect in Cl^- conductance.

Chapter VI

CHARACTERIZATION OF PLATELET-ACTIVATING FACTOR BINDING TO HUMAN AIRWAY EPITHELIAL CELLS: MODULATION BY FATTY ACIDS AND ION-CHANNEL BLOCKERS

Introduction

Platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF) is a potent phospholipid mediator involved in a variety of pathophysiological events, e.g., inflammation, pulmonary and cardiovascular disorders (Braquet et al., 1987; Snyder, 1989; Prescott et al., 1990). PAF is produced endogenously in pathological conditions and also upon stimulation of various cells/tissues (Snyder, 1989; Prescott et al., 1990; Lee, 1987). The effects of extracellular PAF are mediated by interaction of PAF with specific cell membrane receptors (Shukla, 1992; Chao and Olson, 1993). Radioligand binding studies using [^{3}H]PAF have demonstrated specific receptors for PAF in platelets (Valone et al., 1982; Hwang et al., 1983), neutrophils (O'Flaherty et al., 1986; Hwang, 1988), lymphocytes (Travers et al., 1989; Kuruvilla et al., 1991), monocytes (Ng and Wong, 1988), macrophages (Valone, 1988), human lung tissues (Hwang et al., 1985), rat liver tissues (Hwang,1987) and rat brain tissues (Domingo et al., 1988). The PAF receptor from guinea pig lung was recently cloned by functional expression and showed homology to the G protein-coupled receptors with seven transmembrane spanning segments (Honda et al., 1991). The binding of PAF to its receptor initiates a cascade of biochemical events, including phospholipid turnover, activation of protein kinase C, increase in intracellular calcium concentration, generation and release of other mediators (e.g. eicosanoids) (Prescott et al., 1990; Shukla, 1992; O'Flaherty, 1987). Specific PAF receptor antagonists inhibit

PAF-induced responses (Hwang, 1990; Hosford et al.,1989).

PAF has been implicated in the pathogenesis of several pulmonary diseases, and in inflammatory disorders such as asthma (reviewed by Barnes et al., 1989). Pulmonary airway epithelium have been reported to synthesize PAF (Holtzman et al., 1991) as well as eicosanoids (e.g. prostaglandins, leukotrienes, etc.) upon stimulation (Holtzman, 1992), suggesting involvement of the epithelial cells in pathological processes of inflammatory lung disease. Whether or not airway epithelial cells respond to PAF via specific receptors may impact on development of airway inflammation and hypersensitivity because the responses of airway epithelial cells to PAF would mediate and amplify the injury effects of PAF on the inflammed airway via the generation of more mediators. Although a previous study has demonstrated specific binding sites for PAF in human lung tissue homogenates (Hwang et al., 1985), it is unknown if airway epithelial cells express PAF receptors.

Since the effects of extracellular PAF are mediated by interaction of PAF with specific cell membrane receptors (Shukla, 1992), understanding of regulation of PAF receptor and the PAF-receptor-mediated signalling mechanism would provide insight into the pathogenesis and therapy of PAF-involved inflammatory diseases such as asthma. However, knowledge of regulatory factors that affect specific PAF receptors and subsequently control PAF-elicited cellular responses is very limited. Generally, any factor reducing PAF binding to its receptor or subsequent PAF receptor-mediated signal transduction would play a role in anti-inflammation. Fish oil enriched with 20:5ω3 has apparent anti-inflammatory effects (reviewed by Simopoulos, 1991; Sperling, 1991). Although the major mechanism for effect of 20:5ω3 is thought to be a decrease in the production of inflammatory mediators including arachidonate metabolites, PAF and cytokines (Sperling, 1991; Kremer, 1991), the exact mechanism of action of 20:5ω3 is not completely understood. Previous studies found that dietary ω3-polyunsaturated fatty acid supplementation decreased PAF-induced Ca^{++} mobilization and IP_3 formation (Bankey et al., 1989; Sperling et al., 1993), suggesting

an interaction between the fatty acids and PAF's signal transduction. However, whether 20:5ω3 and other fatty acids can affect PAF binding to its receptors remains to be examined.

Pulmonary inflammatory disease is a main manifestation of cystic fibrosis whose basic defect is impermeability of epithelial cell to Cl^- (Quinton, 1983; Collins, 1992). The relationship between the Cl^- impermeability and inflammatory process in the epithelial cell is not known. Whether or not ion conductance across cell membrane can alter inflammatory reactions by affecting the action of inflammatory mediators such as PAF is unknown. Various monovalent and divalent cations have been found to exert regulatory effects on PAF receptor binding (Hwang, 1989). Moreover, cation channel blockers such as Ca^{++}-channel blockers (Valone, 1987; Filep, 1990; Wade et al., 1986) and Na^+-channel blockers (Hwang, 1989) have been shown to inhibit PAF binding to platelets. However, It is unknown whether Cl^- channel blockers affect PAF receptor binding.

The objective of this study is to characterize [^{3}H]PAF binding to human airway epithelial cells and to examine the effects of supplementation of the cell with different fatty acids and ion channel blockers on PAF binding.

Methods

The methods used in this study include cell isolation, primary cell culture, modification of membrane fatty acid composition, gas chromatography, [^{3}H]PAF binding assay, intracellular calcium measurement and are described in detail in Chapter III.

Results

Characterization of [^{3}H]PAF binding to human airway epithelial cells

To examine the kinetics of PAF binding, epithelial cells (1×10^6 cells/ml) were incubated in duplicate with 1 nM of [^{3}H]PAF, with or without 1 μM of unlabelled

PAF for 5, 10, 20, 40, 60 and 120 minutes. Specific binding reached saturation by 30-40 min (Figure VI.1). The kinetics of PAF binding is similar to that found in monocytes (Ng and Wong, 1988) and lymphoblastoid cells (Kuruvilla et al., 1991). Non-specific binding at this concentration (1 nM [3H]PAF) was 50-60% of total binding.

The affinity and number of PAF binding sites in airway epithelial cells was determined by saturation binding assays in which the cells were incubated with

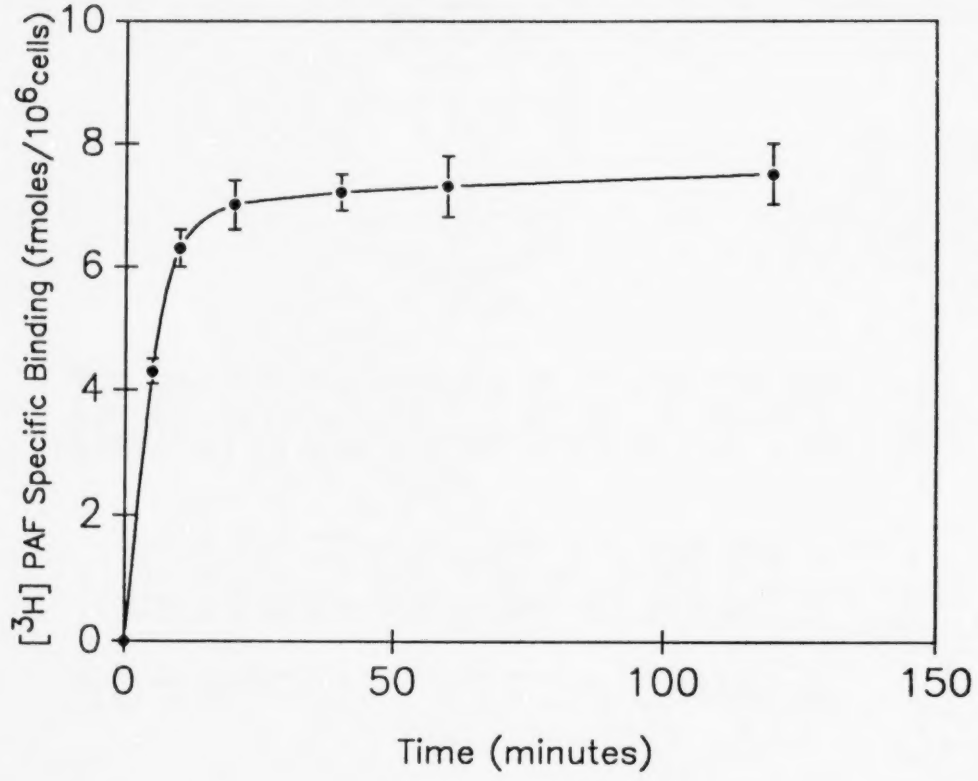

Figure VI.1 Time course of [^{3}H]PAF (1 nM) binding to human airway epithelial cells at 4°C. Specific binding was the difference between PAF binding in the absence and presence of excess unlabelled PAF (1-2 μM). Each value is the mean ± SD of three separate experiments performed in duplicate.

various concentrations of [^{3}H]PAF with or without unlabelled PAF for 60 minutes. Specific binding of PAF to the cells increased with concentration of labelled ligand and the binding was saturable (Figure VI.2). Data from saturation binding was

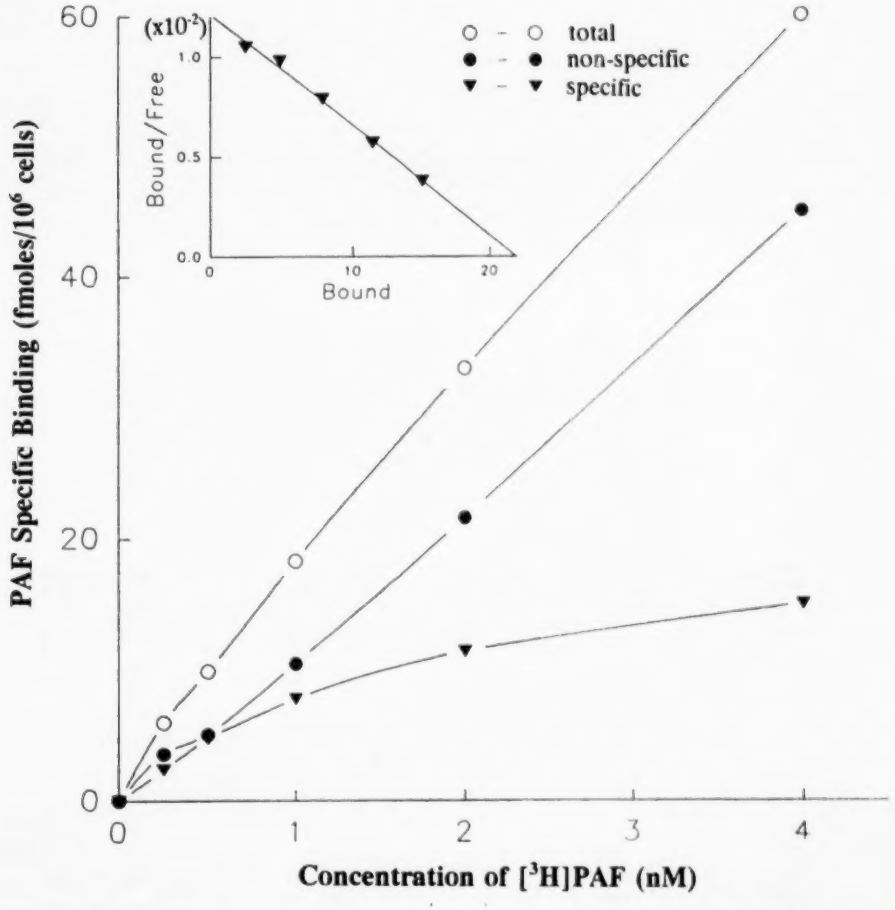

Figure VI.2 Binding isotherm of [^{3}H]PAF to human airway epithelial cells. The cells were incubated at 4°C for 60 min with increasing concentrations of [^{3}H]PAF (0.25-4.0 nM). Specific binding (▲) was calculated as the difference between total (o) and non-specific (•) binding determined in the presence of excess of unlabelled PAF. Inset: Scatchard analysis of the specific binding of [^{3}H]PAF to the epithelial cells. The data are representative of five separate experiments for cells derived from different individuals. The Kd was 1.8 ± 0.2 nM and Bmax was 21.0 ± 2.1 fmole/10^{6}cells.

subjected to Scatchard analysis (Scatchard, 1949). Five separate experiments yielded linear plots (a representative is shown in Figure VI.2 Inset) indicating the presence of a single class of binding sites. The equilibrium dissociation constant representing the affinity (Kd) and the total number of receptor sites (Bmax) are 1.8 ± 0.2 nM and 21 ± 2.1 fmol/10^6 cells (n = 5) , respectively. Assuming an equimolar ligand-receptor complex, the Bmax corresponds to 12,600 ± 1,260 binding sites per epithelial cell.

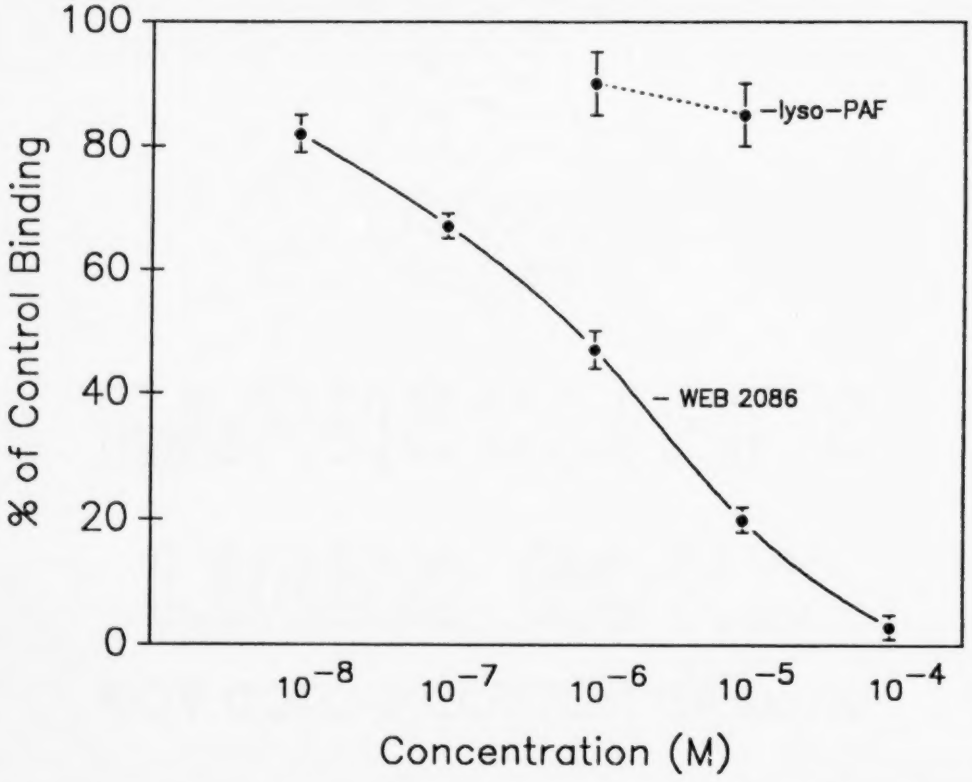

Figure VI.3 Competition of [^{3}H]PAF binding to human airway epithelial cells by PAF receptor antagonist WEB 2086 (●———●) and lyso-PAF (●--●). The cells were incubated with 1 nM [^{3}H]PAF and different concentrations of WEB 2086 and lyso-PAF at 4°C for 60 min. Results are expressed as the mean ± SD of the percent of control binding from 3 separate experiments.

This number of receptors is similar to that found in human monocytes (Ng et al., 1988) and human Raji lymphoblasts (Travers et al., 1989), but greater than that found in neutrophils (O'Flaherty et al., 1986) and platelets (Valone et al., 1982). The Kd for PAF observed in the epithelial cell is very close to that found in human lymphoblasts (Travers et al., 1989; Kuruvilla et al., 1991), higher than that found in human neutrophils (O'Flaherty et al., 1986) and human lung homogenates (Hwang et al., 1985), but lower than that found in human monocytes (Ng and Wong, 1988) and human platelets (Valone et al., 1982).

The specificity of PAF binding was established by means of competition studies utilizing WEB2086, a known potent PAF receptor antagonist (Barnes et al., 1989), and lyso-PAF. WEB2086 inhibited the specific binding of [3H]PAF to the cells in a dose-dependent manner with an IC_{50} = 3-5 x 10^{-7} M, while lyso-PAF at concentration of 10^{-5} M did not apparently inhibit the binding (Figure VI.3), suggesting that the binding sites are specific for PAF.

To assess the functional capability of PAF binding sites, the epithelial cell was loaded with the calcium indicator dye, Fluo-3/AM and the intracellular calcium level was monitored following stimulation of the cell with PAF. PAF at 100 nM induced a rapid rise in intracellular free calcium in epithelial cells (Figure VI.4A). Addition of the PAF antagonist, WEB2086, to the cell suspension 5 min before PAF stimulation resulted in attenuation of the peak response (Figure VI.4B) indicating that the specific sites for PAF are functional receptors. These results are consistent with previous findings in human monocytes (Ng and Wong, 1988), human lymphoblasts (Travers et al., 1989; Kuruvilla et al., 1991) and macrophages (Valone, 1988). A phenomenon of desensitization of $[Ca^{++}]i$ response to PAF was observed (Figure VI.5), suggesting that PAF down-regulates its own receptors (Shukla, 1992).

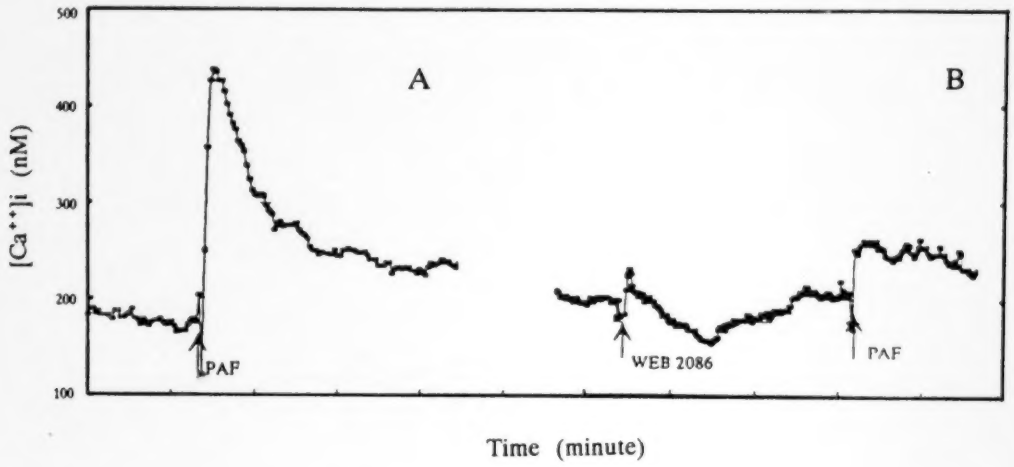

Figure VI.4 Changes in intracellular free calcium induced by PAF (100 nM) in human airway epithelial cells pretreated with solvent vehicle (control, A) or 2 μM of WEB 2086 (B) 5 min before PAF stimulation. Arrows indicate the addition of WEB 2086 and PAF. Results are representative of three separate experiments that produced a change in Ca^{++} concentration of 215 ± 32 nM by 100 nM PAF.

Effect of fatty acid supplementation on PAF binding

To examine the effect of modification of membrane fatty acid composition on PAF binding, 16:0 and 18:2ω6 or 20:5ω3 (100 μM) was added to culture medium and incubated with the cells for 6 h. Supplementation of the cell with individual fatty acids resulted in substantial modification of cellular phospholipid fatty acid composition (Tabel VI.1). Exogenous 20:5ω3 resulted in approximately ten-fold increase in total 20:5ω3 content of phospholipid and a three-fold increase in membrane content of 22:5ω3. Medium supplementation with 16:0 also markedly increased cellular 16:0 and 16:1ω7 content in membrane phospholipid. Consistent with our earlier observations

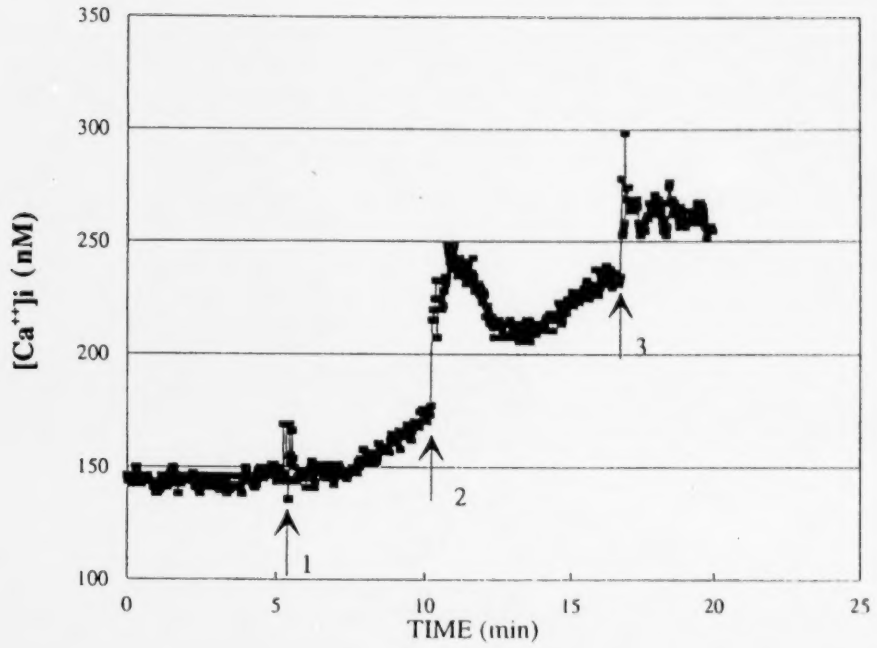

Figure VI.5 Desensitization of $[Ca^{++}]i$ response to PAF. Cultured epithelial cells were loaded with 5 μM of Fluo-3 at 37°C for 40 minutes, then challenged with PAF repeatedly. Change in $[Ca^{++}]i$ was measured by the method described in the text. The arrows indicate addition of vehicle (1), 10nM PAF (2) and 100 nM PAF (3). These results are representative tracings of three experiments.

(Kang et al., 1992), exogenous linoleate (18:2ω6) resulted in a significant increase in the 18:2ω6, 20:3ω6 and 20:4ω6 content in membrane phospholipid.

After incubation with different fatty acids, the cells enriched in each fatty acid were used for assay of PAF-binding . At 1 nM of [^{3}H]PAF, specific binding increased by 50% (from 7.8 ± 0.6 to 11.8 ± 2.5 fmoles/10^6) in cells supplemented with 16:0, but decreased by 65% (from 7.8 ± 0.6 to 2.2 ±1.1 fmoles/10^6 cells) in the cells supplemented with 20:5ω3 . Supplementation with 18:2ω6 did not significantly change PAF binding (Figure VI.6). Scatchard analysis revealed that supplementation

Table VI.1 Effects of fatty acid supplementation on the fatty acyl composition of epithelial phospholipids

Fatty Acid Composition (% w/w)	Fatty Acids Supplemented			
	None	16:0	18:2ω6	20:5ω3
16:0	19.0 ± 0.5	27.0 ± 0.5*	16.4 ± 0.2	17.8 ± 0.2
16:1ω7	4.0 ± 0.4	5.7 ± 0.3*	2.8 ± 0.2	3.6 ± 0.4
18:0	15.5 ± 1.8	15.0 ± 1.5	13.9 ± 0.7	14.0 ± 1.0
18:1ω9	31.5 ± 2.3	22.5 ± 2.0*	19.4 ± 1.1*	22.0 ± 1.2*
18:2ω6	4.5 ± 0.3	4.3 ± 0.1	21.0 ± 0.5*	4.2 ± 0.2
20:3ω6	1.8 ± 0.3	1.9 ± 0.2	2.5 ± 0.3	1.9 ± 0.2
20:4ω6	5.1 ± 0.4	5.5 ± 0.3	6.5 ± 0.3*	4.5 ± 0.3
20:5ω3	0.8 ± o.2	0.8 ± 0.2	-----	8.9 ± 0.3*
22:5ω3	0.7 ± 0.2	0.6 ± 0.1	0.7 ± 0.2	3.1 ± 0.3*

Human airway epithelial cells were cultured for 6 h in medium containing 100 μM exogenous fatty acid. The cells were then harvested, cellular lipids were extracted and fatty acid methyl esters were analyzed by gas-liquid chromatrography as described in the methods section. Values are mean ± SD of three individual cultures (*$P<0.05$).

with 16:0 increased the number of PAF receptors (Bmax: from 26.5 to 42.5 fmoles/10^6 cells) but did not significantly affect affinity (Kd: from 2.2 to 2.7 nM); supplementation with 20:5ω3 reduced the number of PAF receptors (Bmax: from 26.5 to 10.2 fmoles/10^6 cells) without significantly changing receptor affinity (Kd: from 2.2 to 2.9 nM) (Figure VI.7).

To test if the effect of 20:5ω3 is associated with metabolism of the fatty acid, indomethacin, an inhibitor of cyclooxygenase and 5-lipoxygenase (Vanderhoek et al., 1984) was added to medium when cells were incubated with 20:5ω3. Indomethacin

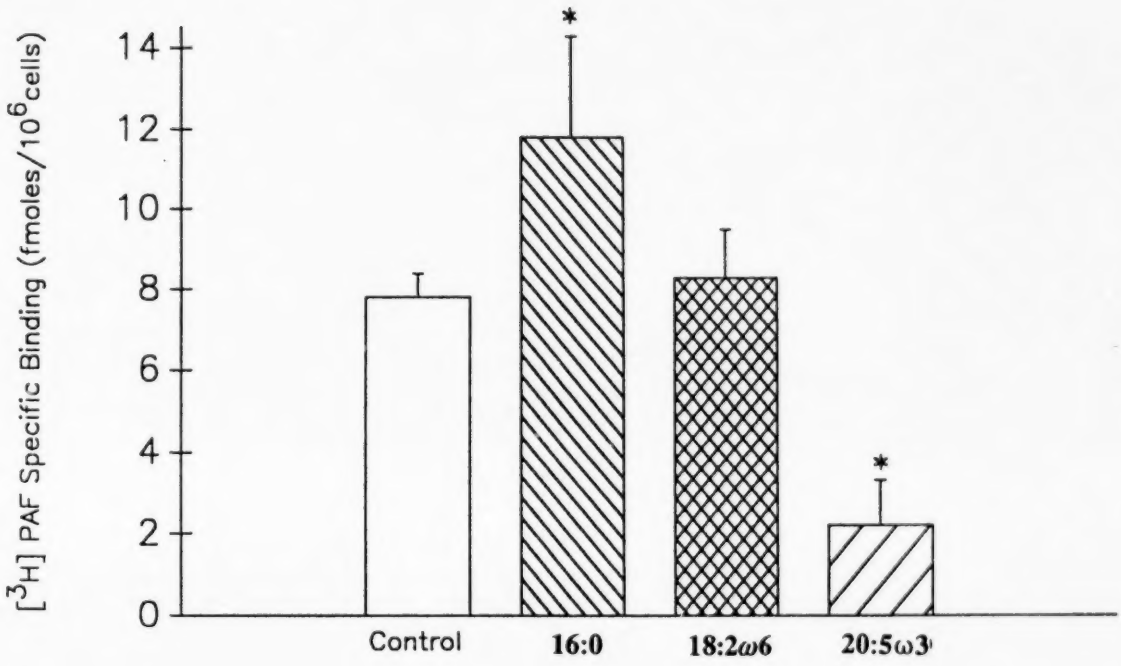

Figure VI.6 Effect of fatty acid supplementation on PAF binding. Epithelial cells were cultured in medium containing 100 μM of different fatty acids for 6 h. The cells were harvested and resuspended in binding buffer and incubated with 1 nM of [^{3}H] PAF in the presence or absence of 1 μM unlabelled PAF for 40 min at 4°C. Specific binding was determined as described in the methods section. Bars represent mean values ± SD of six independent experiments ($p<0.05$).

(100 μM) did not change the effect of 20:5ω3 on PAF binding, suggesting that the effect of 20:5ω3 is not due to its metabolism. Addition of 20:5ω3 to the cells a few minutes before assay of binding had similar but less pronounced effects on PAF binding, indicating that the effect of 20:5ω3 may be partly derived from a direct interaction between unesterified 20:5ω3 and the PAF receptor. The direct interaction between fatty acid and receptor has been previously suggested for steroid receptors (Nunez, 1993).

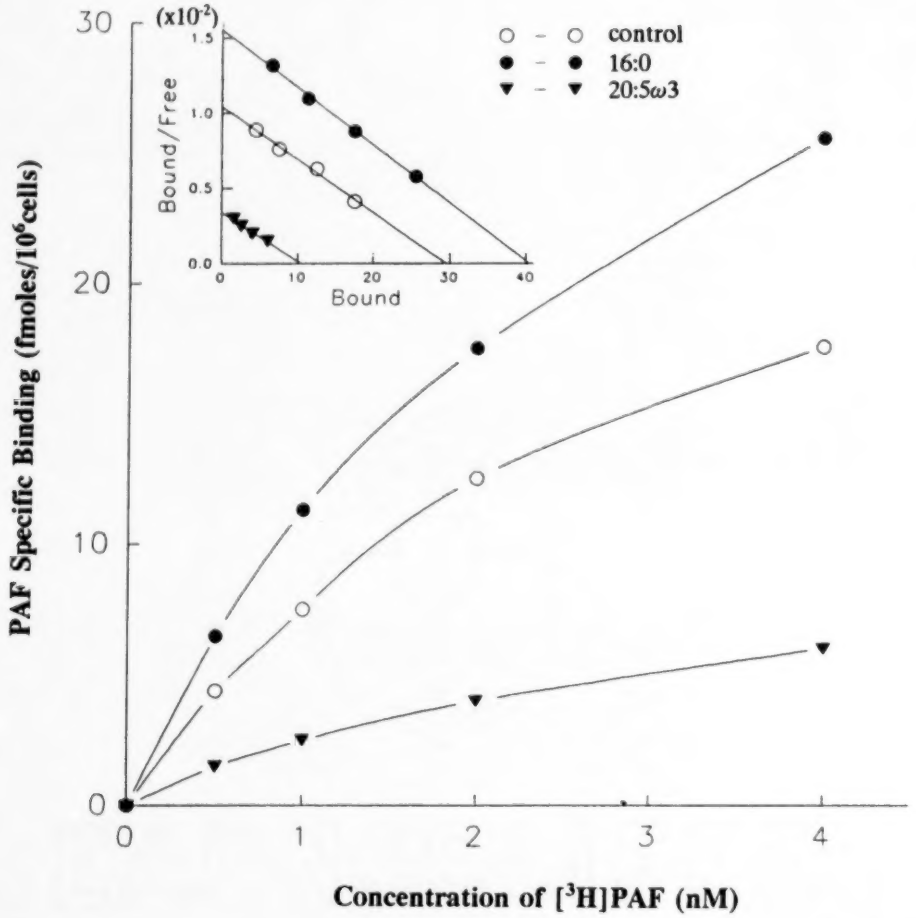

Figure VI.7 Saturation course and Scatchard Analysis of [^{3}H] PAF binding to the cells supplemented with 16:0 or 20:5ω3. Airway epithelial cells were cultured in medium containing 100 μM of 16:0 or 20:5ω3 for 6 h. The cells were then incubated with different concentrations of [^{3}H] PAF in the presence or absence of 1μM unlabelled PAF Specific binding of [^{3}H] PAF was determined as described in the methods section. Values are means of two separate experiments (in duplicate). Inset is a Scatchard analysis of the binding data.

Our preliminary data show that 22:6ω3 and 18:3ω3 also decreased PAF binding by approx. 60% and 25% respectively. It appears that the effect of 18:3ω3 was less potent compared to 20:5ω3 and 22:6ω3.

To test whether 20:5ω3 can suppress PAF-induced Ca^{++} mobilization as a result of decreased PAF binding, changes in intracellular free Ca^{++} concentration in response to different concentrations of PAF was measured after the cells were incubated with or without 20:5ω3 for 5-6 h. Additions of PAF induced typical $[Ca^{++}]i$ response in control cells. Cells supplemented with 20:5ω3 decreased the PAF-

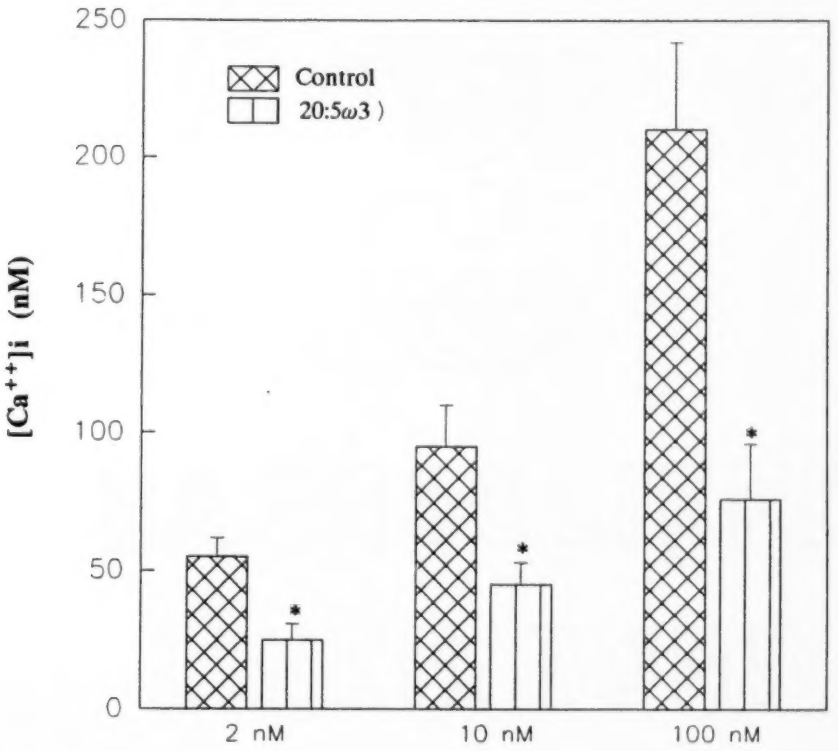

Figure VI.8 Effect of supplementation with EPA on response of $[Ca^{++}]i$ to different concentrations of PAF. Human airway epithelial cells were incubated with and without 100 µM of EPA for 6 h. The cells were loaded with 5µM of Fluo-3 for 40 min. and $[Ca^{++}]i$ was measured as described in the methods section, after stimulation of the cell with 2 nM, 10 nM or 100 nM of PAF. Values are mean ± SD of four experiments.
*$P<0.05$

stimulated increase in $[Ca^{++}]i$ at all concentrations by 50-60% (at 2 nM, from 55 ± 7 to 25 ± 6 (nM); at 10 nM, from 95 ± 15 to 45 ± 8 (nM); at 100 nM, from 215 ± 32 to 76 ± 20 (nM) (n=4)) (Figure VI.8). It seems that the increased $[Ca^{++}]i$ in 20:5ω3-supplemented cells returned to basal-level quickly while the increased $[Ca^{++}]i$ in control cells was sustained above basal levels for a longer time period . It is notable that an apparent relationship between decrease in PAF binding (65%) and reduction in $[Ca^{++}]i$ response (60%) occurs, suggesting that both effects may be related events. It is logical to speculate that the inhibition of PAF-induced increase in $[Ca^{++}]i$ by 20:5ω3 may be the consequence of decreased PAF binding to the cell.

Effects of ion-channel blockers on PAF binding

To assess the effect of Ca^{++} and Na^{+} channel blockers on PAF binding to human airway epithelial cell, different concentrations of verapamil, a Ca^{++}-channel-blocker, or amiloride, a Na^{+}-channel blocker, were added to medium and incubated with cells for 15 min at 37°C, then specific binding of PAF to the cells was assayed at 4°C. It was observed that both verapamil and amiloride inhibited PAF binding, but verapamil was much more potent than amiloride (Figure VI.9). The inhibition of PAF binding by verapamil and amiloride was dose-dependent with a IC_{50} of approximate 4-5 x 10^{-6} M and 2 x 10^{-4} M, respectively (Figure VI.9).. Scatchard analysis revealed that verapamil and amiloride decreased PAF receptor binding affinity and the receptor number (Figure VI.10). These results are similar to those found in previous studies with platelets (Valone, 1987; Wade et al., 1986).

*D*iphenylamine 2-carboxylate (DPC) has been reported to be a Cl^{-} channel blockers in human airway epithelial cells (Welsh, 1986; Gogelein, 1988). To test if DPC affects PAF binding, DPC was added to the cell 15 min before binding assay. DPC increased specific [^{3}H]PAF binding in a dose-dependent manner (Figure VI.11). PAF binding to the cells increased dramatically when DPC concentration was greater

than 100 μM. At 1 mM of DPC, specific binding of PAF was about 3-4 times as much as that in the absence of DPC. Scatchard analysis of PAF binding in the presence of different fixed concentrations of DPC revealed that this agent increased PAF receptor number, but did not affect binding affinity apparently (Figure VI.12).

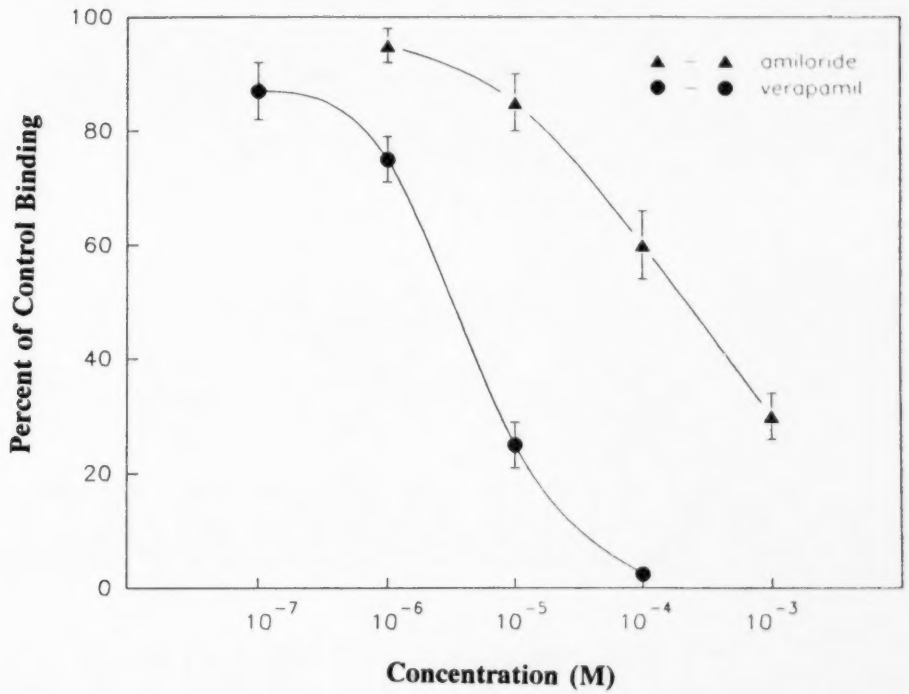

Figure VI.9 Inhibition of specific [^{3}H]PAF binding to human airway epithelial cells by different concentrations of verapamil and amiloride
Cultured epithelial cells (1x10^6 cells) were preincubated with different concentrations of verapamil (dissolved in methanol) or amiloride for 15 min at 37°C, then cooled rapidly to 4°C. Specific binding of [^{3}H]PAF (1nM) to the cells was assayed at 4°C as described in Chapter III. The data point and error bar are the mean and standard deviation of three separate experiments with duplicate determinations.

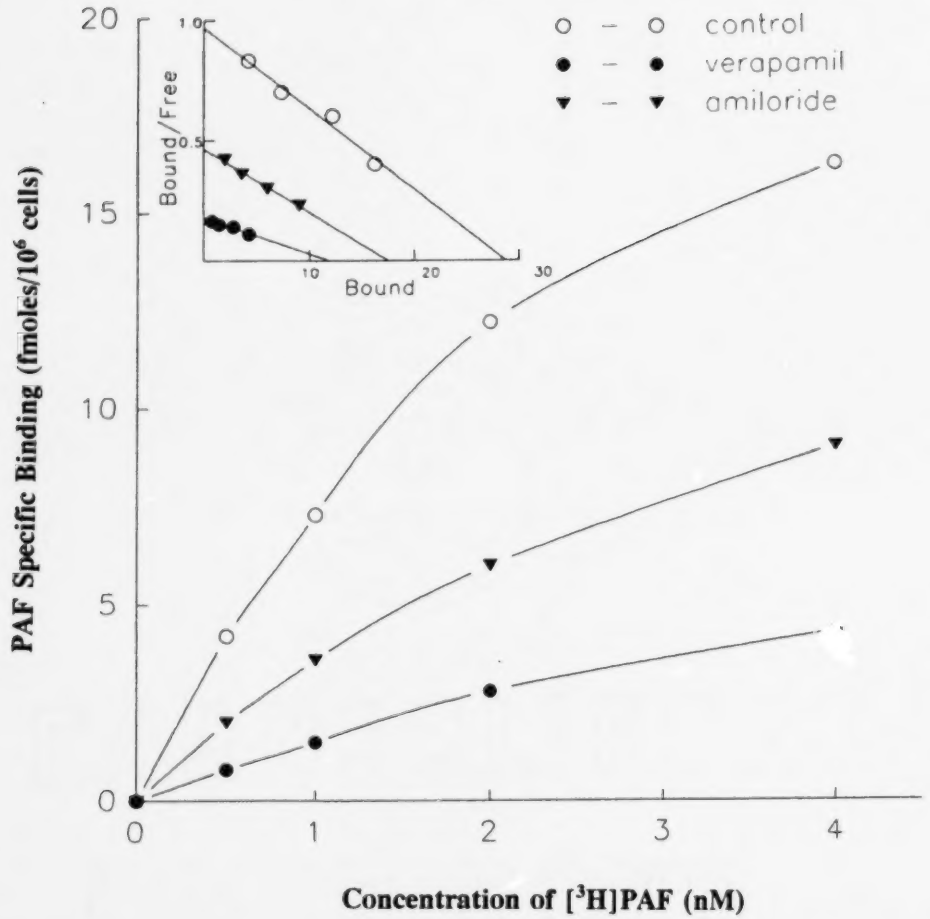

Figure VI.10 Binding isotherm of [^{3}H]PAF to human airway epithelial cells in the presence or absence (control) of ion-channel blockers

Cells ($1x10^6$) were incubated at 4°C for 60 min with increasing concentrations of [^{3}H]PAF (0.5-4nM) in the presence of verapamil (25 μM), (●-●); amiloride (500 μM), (▾–▾) and absence of ion-channel blockers (○-○). Specific binding was determined as described in chapter III. The data are mean of two separate experiments with two determinations. Insert is Scatchard analysis of the binding data.

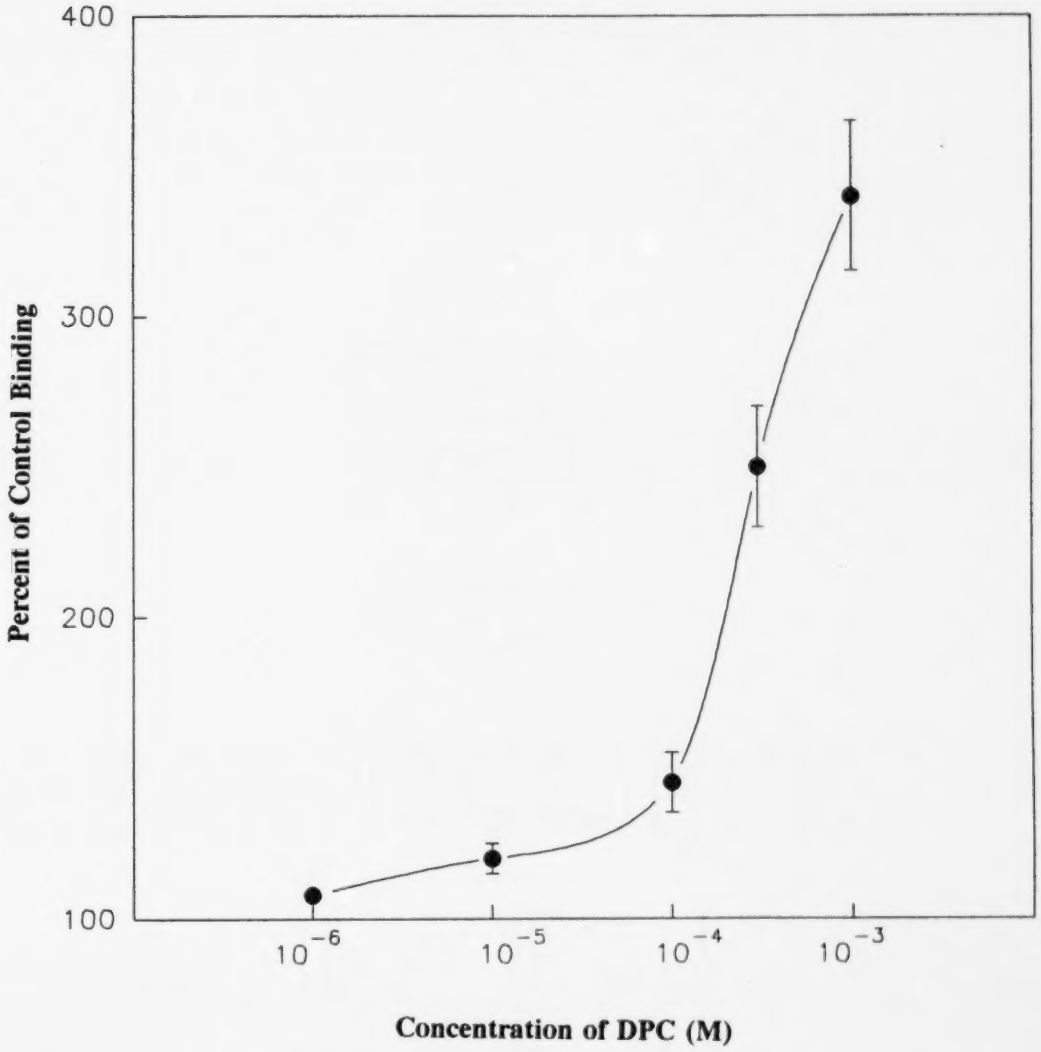

Figure VI.11 Effect of different concentrations of DPC on specific [^{3}H]PAF (1nM) binding to human airway epithelial cells

Cells ($1x10^6$) were preincubated with DPC (dissolved in DMSO) for 15 min at 37°C then cooled rapidly to 4°C. Specific binding of [^{3}H]PAF (1nM) to the cells was assayed as described in chapter III. The data point and error bar are the mean and standard deviation of three experiments with duplicate determinations.

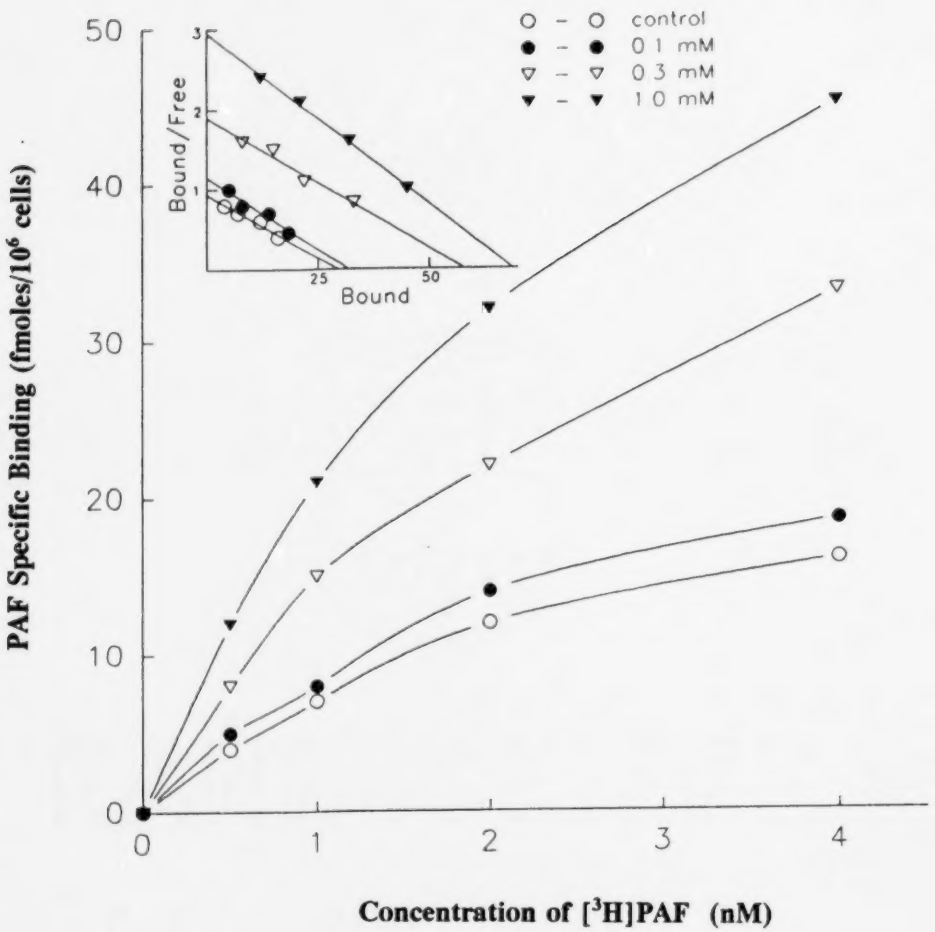

Figure VI.12 Saturation isotherms and Scatchard plots (insert) of [^{3}H]PAF binding to human airway epithelial cells without (○-○) and with DPC at a concentration of 0.1 mM (●-●), 0.3 mM (Δ-Δ) and 1 mM (▲-▲).

Cells (1x10^6) were preincubated with different concentrations of DPC at 37°C for 15 min, then specific binding of increasing concentrations (0.5-4nM) of [3H]PAF was assayed at 4°C. The data are mean of two experiments with duplicate determinations.

Discussion

Characterization of PAF binding

The present study has clearly demonstrated that human airway epithelial cells have functional receptors for PAF. It appears that the cell suspension used in the present study should be an appropriate preparation for study of PAF binding in epithelial cells because it allows exposure of the receptors located in both the apical and basolateral membranes to ligand. Although we can not exclude the possibility that detachment of the cell with trypsinization might damage the receptor, it appears unlikely that the treatment affects binding properties. Previous studies indicate that the ligand binding domain of G protein-coupled receptors is located within the hydrophobic transmembranes core of the receptor protein and that deletion of most the extramembraneous hydrophillic regions of the protein does not affect ligand binding properties, suggesting that the extramembraneous regions of the receptor are not required for ligand binding to occur (Strader et al., 1989). Thus, even if the trypsinization has destroyed some of the extramembraneous regions it would not be expected to change PAF binding activity. In the present study greater levels and higher affinity of PAF binding sites were observed in the epithelial cells compared to some of other human cells.

Existence of functional PAF receptors in human airway epithelial cells suggests that the airway epithelium may play a direct role in the inflammatory process of pulmonary diseases such as asthma and cystic fibrosis. Thus control of PAF receptor expression and/or modulation of PAF binding activity in airway epithelial cells is a novel approach to manipulate pathological process of these pulmonary diseases.

Effects of fatty acid supplementation

It is evident that PAF binding to its membrane receptor can be altered by modifying membrane fatty acid composition. Enrichment of the cell with 20:5ω3

results in a reduction in specific PAF binding and consequently PAF-stimulated increase in intracellular calcium concentration is decreased. These results indicate an interaction between 20:5ω3 and PAF signal transduction pathway.

Previous studies have demonstrated a close association between fatty acids and cell signalling (Sumida et al., 1993) . Free fatty acids have been shown to act as modulators and/or second messengers of signal transduction (Sumida et al., 1993). Fatty acid can directly interact with steroid hormone receptor (Nunez, 1993). 20:5ω3 can decrease binding of angiotensen II receptor (Ullian, 1993), LDL receptor (Saito, 1992), thromobxane A_2 receptor (Swann et al., 1989) and Leukotriene B_4 receptor (Georgilis, 1988), as well as related receptor-mediated responses. In terms of an effect of lipid on the PAF receptor, a previous study (Korth and Middeke, 1991) showed that incubation of monocytic U937 cells with LDL for 24 h increased the number of PAF receptors. In the same cell type another study showed that 22:6ω3 (10 μM) reduced the PAF- induced increase in $[Ca^{++}]i$ (Weber et al., 1991). Dietary ω3 polyunsaturated fatty acid was found to inhibit PAF-mediated formation of IP_3 in human neutrophils (Sperling et al., 1993). Similarly, supplementation with fish oil significantly inhibited both $[Ca^{++}]i$ response and IP_3 formation in response to PAF in rat Kupffer cells (Bankey et al., 1989). Together , these observations and our present findings strongly suggest that there may be an interaction between fatty acids and PAF-mediated signal transduction pathways. Our results further suggest that the effect of ω3 fatty acid on PAF-mediated signal transduction may occur at the ligand-receptor level.

The mechanism by which ω3 fatty acid affects PAF binding activity in the cell is not known. It it possible that the mechanism may involves: direct interaction between 20:5ω3 and the receptor; or 20:5ω3 may bind to the receptor and change receptor conformation, decreasing the availability of binding sites; or change in properties of membrane lipid by modifying membrane lipid bilayer structure, altering receptor

conformation or turnover. It is also conceivable that 20:5ω3 suppresses expression of a gene encoding a receptor component. In addition, it is possible that the decreased PAF binding observed in the cells supplemented with 20:5ω3 is due to blocking a Ca^{++} influx, because Ca^{++} channel blocker was found to decrease PAF binding.

We propose that suppression of signal transduction at the level of PAF-receptor binding and consequently decrease in cellular responsiveness to inflammatory stimuli may provide a novel mechanism by which 20:5ω3 produces an anti-inflammatory effect, prior to its suppression of inflammatory mediator generation.

Effects of ion channel blockers

This study has clearly shown that a calcium channel blocker, verapamil and a sodium-channel blocker, amiloride inhibited PAF binding to human airway epithelial cells, while a chloride-channel blocker, DPC increased PAF binding to the cells. Previous studies have demonstrated that verapamil inhibited PAF binding to human platelets (Valone, 1987; Wade et al., 1986) and human neutrophils (Filep, 1990). Valone (1987) reported that verapamil inhibited PAF binding to human platelets in a dose-dependent manner with 50% inhibition (IC_{50}) at $6.3 \pm 1.2 \times 10^{-5}$ M. Scatchard analysis revealed that verapamil decreased PAF receptor binding affinity, but increased the receptor number in platelets, suggesting that this agent acts in competitive and noncompetitive mechanism. Similar results have been found in human neutrophils except that the IC_{50} is higher ($9.5 \pm 1.4 \times 10^{-5}$ M) (Filep, 1990). Wade et al.(1986) reported that verapamil inhibited PAF binding to human platelets with a IC_{50} of $3.2 \pm 0.7 \times 10^{-5}$ M) and suggested that the effect is competitive based on the observation that the agent decreased PAF receptor binding affinity without affecting receptor number. In the present study, we found that verapamil inhibited

PAF binding to human airway epithelial cells with a IC_{50} of 4-5 x 10^{-6} M; Scatchard analysis of PAF binding in the presence of verapamil indicated that this agent decreased both PAF receptor binding affinity and PAF receptor number. This discrepancy in potency and mechanism of action of verapamil observed in different studies may be attributed to differences in receptors in different cell types and / or differences in the experimental conditions. These results that both affinity and binding site were altered by verapamil support the suggestion that the mechanism by which calcium channel blockers inhibit PAF binding is probably more complex than simple competition for receptor binding. It is possible that blocking Ca^{2+} influx and subsequently changing intracellular Ca^{++} concentration by Ca^{++} channel blocker contribute, at least in part, to its effect on PAF binding. PAF binding to its receptor may be depend on intracellular Ca++ concentration, since increase in extracellular Ca^{++} concentration enhances PAF binding (Hwang et al., 1986). It is also possible that a Ca^{++} channel is coupled with the PAF receptor and regulates the receptor binding activity. However, the exact mechanism by which Ca^{++}-channel blockers inhibit PAF binding remains to be studied.

Similar to the Ca^{++}-channel blocker, amiloride, a Na^{+}-channel blocker, also has inhibitory effects on PAF binding but it was less effective compared to verapamil. This result is consistent with previous findings that amiloride and its analogues inhibit the specific PAF binding to rabbit platelet membrane (Hwang, 1989). Amiloride has been proven to be an effective drug in improving pulmonary pathological conditions of cystic fibrosis. The effect of amiloride, may be in part due to its inhibitory effect on PAF binding in addition to inhibition of Na^{+} and water absorption.

In terms of the effect of Cl^{-} channel blocker on PAF binding, there is no relevant information available so far. In the present study, it was observed that 1 mM DPC, which has been shown to inhibit Cl^{-} conductance across membrane in airway epithelial cells at this concentration (Welsh, 1986), increased specific binding of PAF to the epithelial cells by 2-3 fold. How blocking the Cl^{-}-channel increases PAF binding

in the epithelial cell is not known. We speculate that blocking Cl^--channel leads to change in intracellular Cl^- concentration, which may cause alteration of cellular volume, cellular PH and membrane potential. These changes may activate gene expression and protein modification perhaps stimulate cellular protein translocation between cellular compartments, for example, transfer protein from endoplasms reticulum to plasma membrane, consequently result in higher density of PAF receptor in cell membrane. However, it can not be excluded that the effect of DPC is due to direct interaction between DPC and membrane lipid and/or membrane protein (PAF receptor) rather than blocking Cl^--conductance.

In summary, these results imply that ion channel function may modify the action of PAF at the receptor level. The present finding that blocking the Cl^--channel increases PAF binding may have physiological relevance to pathological conditions such as cystic fibrosis in which both Cl^- impermeability and inflammation occur. It is possible that the defect in Cl^- transport potentiates the inflammatory reaction in CF. Furthermore, it is suggestive that since Ca^{++} and Na^+-channel blockers reduce PAF binding to epithelial cells, they may be beneficial drugs for treatment of PAF-involved inflammatory diseases.

Chapter VII

MODULATION OF PLATELET ACTIVATING FACTOR GENERATION BY EXOGENOUS FATTY ACIDS IN HUMAN LUNG EPITHELIAL CELLS (A549)

Introduction

Platelet-activating factor has been implicated in the pathogenesis of inflammatory diseases in the lung and elsewhere (Barnes et al., 1989; Smith, 1991). PAF produces an inflammatory response in the airways of several species including humans (Camussi et al., 1983; Arnoux et al., 1988; Wardlaw et al., 1990), since PAF is a potent chemotaxin for human neutrophils (Wardlaw et al., 1986) as well as a potent bronchoconstrictor (Vargaftig et al., 1980; Rubin et al., 1987). The inflammatory effects of PAF on airway induce an increase in airway mucus secretion (Wirtz et al., 1986) and a decrease in mucociliary clearance (Aursudkij et al., 1987), and ultimately produce a sustained increase in airway responsiveness and airway damage (Chung et al., 1986; Kaye and Smith, 1990).

PAF is synthesized in response to specific stimuli by a variety of cells, including human pulmonary epithelial cells (Hassan and Wong,1990, Hotzman et al., 1991) Agonist-stimulated synthesis of PAF occurs in a two step pathway involving the deacylation of membrane 1-O-alkyl-2-acyl-glycerophosphocholine (1-alkyl-2-acyl-GPC) by phospholipase A_2 and the subsequent acetylation of 1-alkyl-2-lyso-GPC by acetyl-CoA acetyltransferase (Snyder, 1989). As a consequence of the deacylation of alkyl arachidonyl-GPC, free arachidonic acid (20:4ω6) is released and converted into eicosanoids, particularly, leukotriene B_4 (Chilton and Lichtenstein, 1990), which is a potent chemoattractant and has many important effects on leukocyte functions relevant to the mediation of airway inflammation (Henderson, 1987).

Previous studies have suggested that PAF and eicosanoid biosynthesis may be

tightly coupled because both precursors of PAF and eicosanoids can be mobilized from a common precursor molecule, 1-O-alkyl-2-arachidonoyl-GPC by the action of a phospholipase A_2 during cell activation (Chilton and Lichtenstein, 1990). It has been shown that PAF can induce synthesis of leukotrienes, prostaglandins and thromboxanes in a number of systems (Shaw et al., 1981; Chilton et al., 1982; Yousufzai, 1985) Conversely, some lipooxygenase products have been shown to increase PAF biosynthesis in human neutrophils (Billah et al., 1985; Tesser et al, 1989). It seems that a complex system of regulatory and feedback mechanisms exists for PAF and arachidonic acid metabolites in the organs and cells that produce them. However, the precise relationship and interaction between PAF and eicosanoid production in a particular cell type or tissue remains to be examined.

Airway epithelial cells have been found to produce PAF and eicosanoids (Holtzman, 1992) suggesting that the epithelial cell plays an important role in inflammatory processes of pulmonary disease such as asthma and cystic fibrosis. Thus, control of PAF and eicosanoid production by the lung epithelial cell is of considerable pathophysiological importance. Little is known about the factors that regulate PAF biosynthesis in pulmonary epithelial cells. This study examines the effect of supplementation of the epithelial cell with different fatty acids on PAF generation and whether eicosanoids are able to induce PAF production in a human lung epithelial cell line (A549) that biochemically and functionally resembles bronchial type II epithelial cells (Lieber et al., 1976) and has the capability to produce PAF (Hassan and Wong, 1990).

Methods

The methods used in this study include cell line culture, modification of membrane fatty acid composition, lipid analysis, radioimmunoassay and are described in detail in Chapter III.

Results

Effect of fatty acid supplementation on the fatty acyl composition of epithelial phospholipid.

To modify fatty acid composition of cellular phospholipid, 100 μM of 16:0, 18:1ω9, 18:2ω6, 20:4ω6 or 20:5ω3 was added to the culture medium and incubated with the cell for 6 h. Lipid analysis indicated that each fatty acid was effectively incorporated into cellular phospholipids, resulting in significant change in fatty acid

Table VII.1 Effect of fatty acid supplementation on membrane fatty acid composition of A549 cells

Fatty acid composition (w/w)	Fatty acids supplemented					
	Control	16:0	18:1ω9	18:2ω6	20:4ω6	20:5ω3
15:0	2.8 ± 1.0	1.4 ± 0.2	2.7 ± 0.5	1.8 ± 0.7	1.7 ± 0.4	2.8 ± 0.8
16:0	19.6 ± 1.7	25.3 ± 2.0*	16.3 ± 1.5	17.1 ± 1.6	21.2 ± 0.1	17.2 ± 1.3
16:1ω7	3.5 ± 0.4	2.8 ± 0.3	3.4 ± 0.4	3.4 ± 0.6	4.7 ± 0.1	3.3 ± 0.4
16:1ω5	3.8 ± 0.5	3.1 ± 0.3	3.9 ± 0.1	3.7 ± 0.7	5.0 ± 0.1	3.6 ± 0.3
18:0	15.8 ± 1.2	16.5 ± 0.3	15.0 ± 3.0	12.9 ± 0.8	12.3 ± 0.2	13.2 ± 0.7
18:1ω9	31.6 ± 1.5	29.3 ± 0.8	41.9 ± 0.7*	23.6 ± 1.2	24.2 ± 0.2	25.8 ± 1.4
18:1ω7	7.4 ± 0.4	6.3 ± 0.1	5.4 ± 0.2	5.2 ± 0.3	5.7 ± 0.2	6.0 ± 0.3
18:2ω6	1.8 ± 0.2	1.8 ± 0.1	1.4 ± 0.0	19.2 ± 1.2*	1.5 ± 0.0	1.6 ± 0.3
18:3ω6	---	---	---	0.5 ± 0.1	---	---
20:1ω9	---	---	0.5 ± 0.1	---	---	---
20:2ω6	1.8 ± 0.3	1.9 ± 0.2	1.4 ± 0.2	1.5 ± 0.2	1.7 ± 0.5	1.3 ± 0.1
20:3ω9	0.3 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	---	0.3 ± 0.1
20:3ω6	0.9 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
20:4ω6	6.5 ± 0.7	7.9 ± 0.8	5.1 ± 0.2	5.5 ± 0.2	14.7 ± 0.1*	5.7 ± 0.2
20:5ω3	1.1 ± 0.2	1.2 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	12.0 ± 1.5*
22:4ω6	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.8 ± 0.2	0.3 ± 0.1
22:5ω6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
22:5ω3	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	1.0 ± 0.1
22:6ω3	1.2 ± 0.1	1.2 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	1.0 ± 0.1
ΣSFA	38.2 ± 1.5	43.0 ± 1.7*	35.0 ± 1.7	32.9 ± 2.0	38.6 ± 0.1	34.5 ± 2.9
ΣMUFA	47.4 ± 0.7	41.9 ± 0.7*	54.4 ± 2.8*	37.0 ± 0.6*	41.1 ± 0.2*	40.0 ± 1.2*
Σω6FA	11.4 ± 1.4	13.1 ± 1.2	8.7 ± 0.6	27.8 ± 2.1*	18.6 ± 0.1*	10.4 ± 0.7
Σω3FA	2.5 ± 0.4	2.6 ± 0.3	1.8 ± 0.5	1.9 ± 0.2	1.7 ± 0.2	15.0 ± 3.0*

A549 cells were incubated with the indicated fatty acids for 6 h. Phospholipid fatty acid composition was quantified by capillary gas liquid chromatography as described in chapter III. The data represent means ± SE of four seperate determinations. *P<0.05 vs control.

composition (Table VII.1). Exogenous arachidonate resulted in a 2-fold increase in cellular 20:4ω6 (from 6.5 to 14.7%). Supplementation with 20::5ω3 and 18:2ω6 increased their content in phospholipid by approx 10-fold, while supplementation with palmitate had only a modest effect on cellular 16:0. No significant increase in respective elongation/desaturation products of these supplemented fatty acids was observed in this cell line.

Effect of fatty acid supplementation on PAF synthesis by lung epithelial cells

After incubation with different fatty acids, cells were washed and stimulated with 2.0 μM ionophore A23187 for 10 minutes based on previous findings that the synthesis of PAF by this cell line through ionophore A23187 (1-2 μM) stimulation reached a maximum at about 5-10 min (Hassan and Wong, 1990). Cell-associated PAF and PAF released was measured using radioimmuoassay (RIA). Stimulation of cells with the calcium ionophore resulted in significant PAF production (8.2 pmoles/10^6 cells) (Figure VII.1). Unstimulated cells synthesized only small amounts of PAF (0.8-1 pmoles/10^6 cells). Supplementation with 20:4ω6 significantly enhanced agonist-stimulated synthesis of PAF in the epithelial cell. (from 8.2 $\pm$ 0.6 to 11.0 $\pm$ 0.5 pmoles/10^6 cell, $p < 0.05$). This finding is similar to previous observations in endothelial cells (Carcia et al., 1991) and human promyelocytic leukemia (HL-60) cells (Suga et al, 1990). Supplementation with 20:5ω3 decreased PAF production rather than increased PAF generation as found in endothelial cells (Carcia et al., 1991) and HL-60 granulocytes (Suga et al., 1990), though the difference was not significant. This result is consistent with similar observations for the human monocyte (Sperling et al., 1987). Supplementation with 18:1ω9 also slightly decreased PAF synthesis in the cells. Supplementation with 16:0 and 18:2ω6 did not affect PAF generation. Consistent with a previous study (Hassan and Wong, 1990) the amount of PAF released to the medium was about 20-30% of the total PAF produced by the

cells, suggesting that the majority of PAF produced is retained by the cells. Percentage release of PAF was not affected by supplementation with different fatty acids.

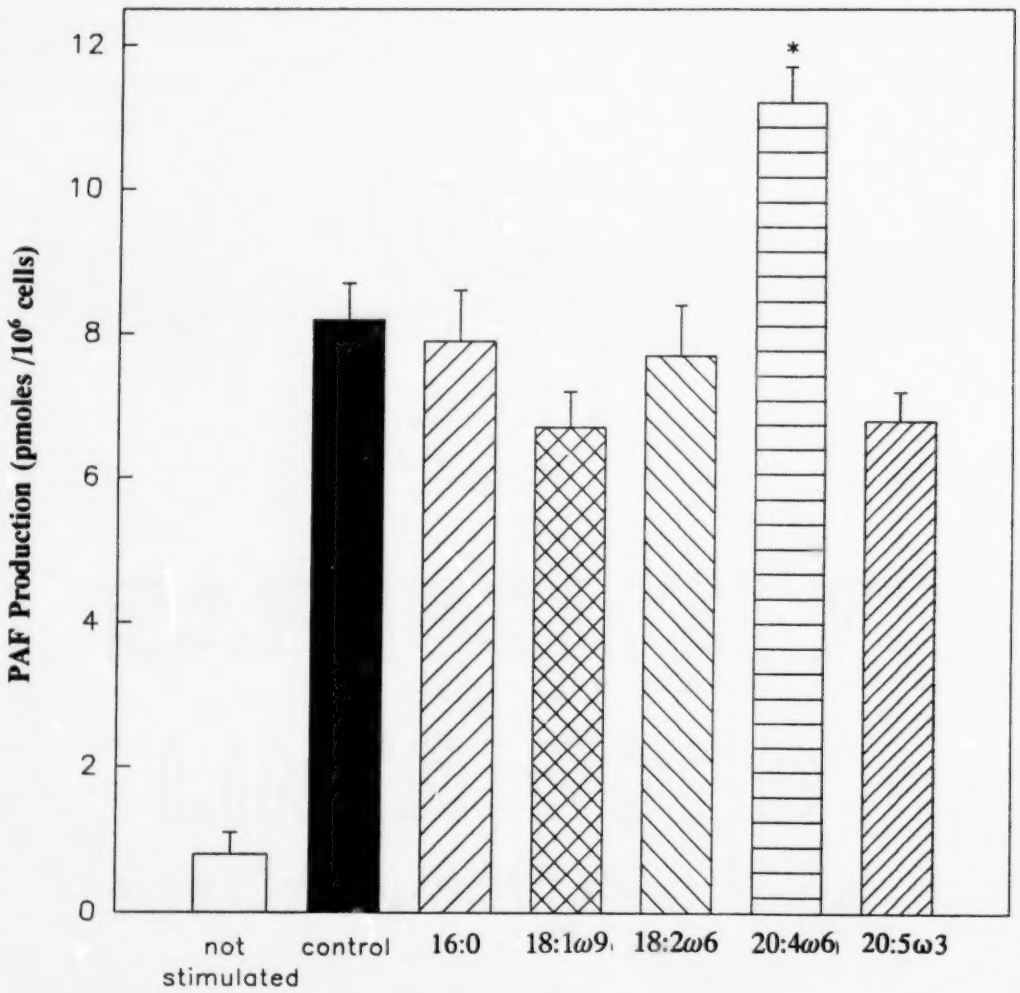

Figure VII.1 Effect of fatty acid supplementation on PAF generation by A549 cells After incubation with various fatty acid (100 μM) for 6 h, A549 cells were stimulated with 3 μM A23187 for 10 min. PAF was measured by radioimmunoassay as described in chapter III. The data represent mean ± SE of four separate experiments with duplicate determinations. *P<0.05 vs control.

Effect of arachidonic acid metabolites on PAF synthesis.

To test whether eicosanoids can directly stimulate synthesis of PAF, 5 μM of PGE_2 , $PGF_{2\alpha}$, LTB_4 , LTD_4 or LTE_4 was added to medium and incubated with the cells for 15 min. PAF generation was determined by radioimmuoassay. The

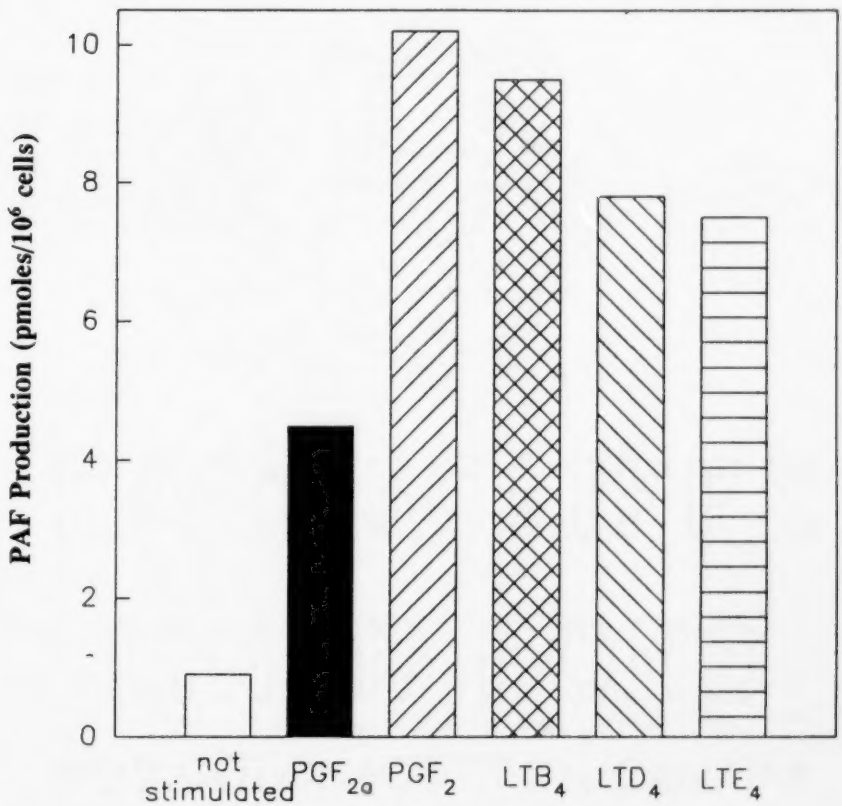

Figure VII.2 Effect of eicosanoids on PAF Production
A549 cells were stimulated with one of the indicated eicosanoids (3 μM) for 15 min, then the PAF generated by the cell was measured by radioimmunoassay. The data are mean of two separate experiments with duplicate determinations. (variation < 10%).
PG, prostaglandin; LT, leukotriene.

amount of PAF found in the cells incubated with eicosanoids was 5-10 fold more than that found in unstimulated cells (Figure VII.2). Obviously, these compounds all independently can stimulate PAF production without a calcium ionophore. PGE_2 and LTB_4 are more potent agonists than $PGF_{2\alpha}$, LTD_4 and LTE_4 (Figure VII.2). This result is partly consistent with previous studies in which LTB_4 was shown to modulate PAF synthesis in human neutrophils (Billah et al., 1985; Tesser et al., 1989), but contrary to the previous observation that LTB_4 alone was without effect in human neutrophils (Billah et al., 1985). The present study also suggests that other eicosanoids can stimulate PAF synthesis.

Discussion

This study confirms that the human lung epithelial cells (A549) synthesize PAF and suggests that synthesis of PAF by epithelial cells can be modulated by modification of the fatty acid composition of the cellular phospholipid. Specifically, enrichment of the lung epithelial cells with 20:4ω6 significantly enhances agonist-stimulated synthesis of PAF , while exogenous 20:5ω3 slightly decreased generation of PAF. The present study also demonstrates that arachidonate metabolites were potent stimuli of PAF formation, thereby suggesting an interaction between PAF and eicosanoid production in the cells.

Previous studies strongly suggest that 1-alkyl-2-arachidonoyl-glycero-3-phosphocholine (1-alkyl-2-arachidonoyl-GPC) is a common precursor for PAF and eicosanoids (Chilton and Lichtenstein 1990; Alberta and Snyder 1984) and that an arachidonate-specific phospholipase A_2 is responsible for catalyzing the initial step for PAF production in the remodelling pathway (Suga et al., 1990) so that arachidonic acid is tightly associated with PAF generation. The present observation (Table VII.1 and Figure VII.1) that supplementation of lung epithelial cells with arachidonic acid increases PAF production supports this suggestion. It is possible that supplementation of epithelial cells with arachidonate increases the pool of alkyl

arachidonoyl-GPC which is hydrolysable by an agonist-stimulated arachidonoyl-specific phospholipase A_2 and is thus available for subsequent acylation to produce PAF. Similar effects of arachidonate supplementation on PAF synthesis has been observed for vascular endothelial cells (Carcia et al., 1991) and for monocytes (Sperling et al., 1987). Thus it appears that availability and / or pool size of alkylacyl-GPC, especially alkylarachidonoyl-GPC, is important in the synthesis of PAF.

In addition to its effect on the availability of alkylarachidonoyl-GPC, arachidonic acid may affect synthesis of PAF through its metabolites. Although Suga et al. (1990) reported that the effect of arachidonate supplementation on PAF synthesis by HL-60 granulocytes was relatively unaffected by cyclooxygenase and / or lipoxygenase inhibitors, it has been suggested that lipoxygenase products of arachidonate regulate PAF biosynthesis (Billah et al., 1985; Tesser et al., 1989). Billah et al. (1985) reported that in human neutrophils, exogenous 5-HETE and LTB_4 stimulate PAF formation. Similar results have been found by Tesser et al. (1989) in the same cell type. The present findings (Figure VII.2) confirm that LTB_4 can stimulate PAF production and extends observations found previously in human neutrophils to human lung epithelial cells and further suggests that not only LTB_4 but many other eicosanoids also stimulate PAF formation. Although the mechanism by which eicosanoids stimulate PAF synthesis is not well understood, there are at least two possibilities. Eicosanoids enhance the expression of phospholipase A_2. This mechanism is suggested by a previous study (Billah et al., 1985) in which LTB_4 or 5-HETE synergistically promoted A23187-stimulated PAF synthesis, but these metabolites were ineffective in the absence of ionophore A23187. Secondly, eicosanoids may bind to their receptors or other membrane protein to induce a rise in cytosolic calcium concentrations, or to activate protein kinase C which in turn, activate phospholipase A_2 and/or acetyltransferase. In this case, exogenous eicosanoids can immediately stimulate PAF production without ionophore A23187. This phenomenon was observed in the present study (Figure VII.2) and previous

work (Tesser et al., 1989).

In summary, enhancement of agonist-stimulated synthesis of PAF by supplementation with 20:4ω6 as observed in this study can be explained by two different mechanisms: 1) an increase in availability and/or pool size of alkylarachidonoyl-GPC; and 2) through the action of eicosanoids derived from the arachidonate supplemented. Thus it is conceivable that any fatty acid that can interact with these pathways could affect PAF synthesis.

Previous results on the effect of eicosapentaenoate (20:5ω3) supplementation on PAF formation are controversial. Studies using HL-60 granulocytes (Uga et al., 1990) and endothelial cells (Carcia et al., 1991) showed that enrichment of cellular phospholipid with 20:5ω3 enhanced PAF synthesis. By contrast, Sperling et al (1987b) found that dietary supplementation with fish oil decreased monocyte synthesis of PAF; enrichment of monocytes in vitro with 20:5ω3 was also inhibitory (Sperling et al., 1987a). A similar result was observed in renal microsomes from rats fed a 20:5ω3-enriched diet (Yeo and Holub, 1989). Consistent with the latter studies, the present results (Fig.VII.1) showed that supplementation with 20:5ω3 slightly decreased PAF formation. It seems unlikely that decreased synthesis of PAF by 20:5ω3-supplemented cells is due to replacement of 20:4ω6 by 20:5ω3, because the 20:4ω6 content of phospholipid was not changed by supplementation with 20:5ω3. Thus, it is possible that the effect of 20:5ω3 on PAF synthesis is the consequence of inhibition of eicosanoid synthesis from 20:4ω6 by 20:5ω3. It has been evident that 20:5ω3 can diminish eicosanoid, particularly LTB_4 synthesis (Sperling, 1991). It is also possible that the effect of 20:5ω3 on PAF synthesis may be via the inhibition of acetyltransferase activity (Yeo and Holub, 1989). It was observed that 18:1ω9 also had an inhibitory effect; perhaps this fatty acid can also interfere with eicosanoid synthesis, because its elongation/desaturation product 20:3ω9 can inhibit leukotriene

A hydrolase (Stenson et al., 1984). Another explanation for the effect of 18:1ω9 is that phospholipase A_2 may be less active (specific) to alkyl-oleatyl-GPC species.

The present observation that eicosanoids were able to induce PAF production, together with previous findings that PAF stimulated eicosanoid generation (Shaw et al., 1981; Chilton et al., 1982; Yousufzai, 1985; Billah et al., 1985; Tesser et al., 1989), suggests that autacoids (PAF and eicosanoids) are linked in a network where various elements interact to promote their mutual production and bioactions. Through this network, a single agonist can initiate the synthesis of a cascade of mediators and its own further synthesis. This amplifies mediator production, ensures the synchronous formation of synergistically interacting mediators and may allow a stimulus to elicit responses via the effectors it induces. Such an intricate network may be critical for orchestration of complex in vivo reactions during inflammatory process. Further study to better define the interaction between the individual molecules of the network is essential to understanding of role of lipid mediators in the inflammatory diseases.

Since interaction between lipid mediators can amplify inflammatory response, identification of the key mediators and understanding of their regulation in an inflammatory disease is important for therapy of the disease. A most recent study (Lawrence and Scorell, 1993) suggests that LTB_4 is involved in the pathogenesis of destructive lung disease in cystic fibrosis and has shown a potential way of reducing this effect in vivo by dietary supplementation with 20:5ω3. The present observation that LTB_4 is a potent agonist for PAF generation (Figure VII.2) and supplementation of cells with 20:5ω3 decreases PAF generation (Figure VII.1) and PAF binding (chapter VI) in human lung epithelial cells are consistent with a role for LTB_4 in the inflammatory response to chronic lung infection and a beneficial effect for 20:5ω3 in anti-inflammation. Thus it is implied that dietary supplementation with 20:5ω3 may be a new therapeutic approach to cystic fibrosis.

Chapter VIII

GENERAL SUMMARY AND DISCUSSION

General Summary

Essential fatty acid metabolism in human airway epithelial cells has been characterized (Chapter IV). Primary cultured human airway epithelial cells were incubated with [1-^{14}C]-18:2ω6 (1 μCi, 100 nmoles) to determine the capacity of the epithelial cell to incorporate and desaturate/elongate 18:2ω6. Following 4 h incubation, 25-30% of the [^{14}C]18:2ω6 supplemented to culture medium was incorporated into the cell with 60-70% in membrane phospholipid (mostly in PC) and 20-30% in triglyceride, indicating that cultured human airway epithelial cells rapidly and effectively incorporate exogenous fatty acids into cellular lipids (hypothesis 1); Thirty percent of 18:2ω6 incorporated was rapidly converted to homologous trienes, tetraenes and pentaenes, the major products being 20:3ω6 and 20:4ω6, suggesting that the cells have capability to desaturate and elongate exogenous fatty acids (hypothesis 2); The conversion of 18:2ω6 was time-dependent and donor age-related. A higher proportion of 20:3ω6 and 20:4ω6 was incorporated into phosphatidylinositol and phosphatidylethanolamine. About 10-15% of total product formed from 18:2ω6 was released from the cell to the culture medium. Both 20:4ω6 and 20:5ω3 inhibited 18:2ω6 incorporation and desaturation. With pulse-chase studies, the half life for 18:2ω6 in phospholipid was estimated to be 5-7 h. The rate of fatty acid turnover in individual phospholipids was in the order: PC > PI > PE > PS. This indicates that fatty acyl chains of the epithelial phospholipid are in a dynamic state of rapid turnover (hypothesis 3); As a result of rapid turnover of phospholipid fatty acyl chains, the fatty acid composition of the epithelial phospholipid was substantially changed following 4 h supplementation with fatty acid to culture medium, suggesting

that essential fatty acid profile of the cells is readily and rapidly modified by exogenous fatty acids (hypothesis 4). Overall, these data demonstrate active metabolism of essential fatty acids in human airway epithelial cells.

The effect of a chloride channel blocker on fatty acid incorporation and desaturation in cultured human airway epithelial cells was examined (Chapter V). Anthracene-9-carboxylate (9-AC) reduced 18:2ω6 incorporation into phospholipid by 60-70% and increased incorporation of 18:2ω6 into triglyceride by 50-100% under conditions known to inhibit Cl^- conductance across epithelial membranes. The decrease in 18:2ω6 incorporation into phospholipid was rapid and dependent on the concentration of 9-AC. Substitution of extracellular Cl^- with gluconate significantly decreased 18:2ω6 incorporation into phospholipid, suggesting that the effect of 9-AC may occur by inhibiting Cl^- conductance. The effect on 18:2ω6 incorporation could not be induced by other ion-channel blockers such as amiloride, a Na^+-channel blocker or verapamil, a Ca^{++}-channel blocker. Incorporation of the non-essential fatty acids, 18:1ω9 and 16:0, into phospholipid was less affected by 9-AC when compared to 18:2ω6. Lipid analysis of cells exposed to 50 μM 18:2ω6 revealed that the level of 18:2ω6 in cell membrane phospholipid was significantly lowered as a consequence of the effect of 9-AC. The relative rate of 18:2ω6 desaturation was not apparently changed by 9-AC. These data indicate that blocking the Cl^- channel alters the essential fatty acid profile of the epithelial cells by reducing incorporation into phospholipid (hypothesis 5).

Specific [^{3}H]PAF binding to human airway epithelial cells has been characterized (Chapter VI). Direct radioligand binding studies were performed in primary cultured human airway epithelial cells with [^{3}H]-PAF. Specific binding of PAF reached saturation within 30 minutes. Scatchard analysis of PAF binding data revealed a single class of PAF binding sites with a Kd of 1.8 $\pm$ 0.2 nM and Bmax of

21.0 ± 2.1 fmol/10^6 cells (13,000 receptors/cell) . WEB2086, a potent specific PAF receptor antagonist, displaced the binding of [^{3}H]PAF to the cell. PAF binding increased the intracellular free calcium concentration of the cells and this effect was inhibited by WEB2086, indicating that human airway epithelial cells have functional PAF receptors (hypothesis 6).

To test the effect of supplementation of the cells with different fatty acids on PAF binding, 100 μM of palmitate (C16:0), linoleic acid (18:2ω6) or eicosapentaenoic acid (20:5ω3) was incubated with the cells for 6 h and then fatty acid composition, PAF binding and [Ca^{++}]i were measured (Chapter VI). Lipid analysis revealed effective incorporation of each fatty acid into cellular phospholipid. [^{3}H]PAF (1 nM) binding decreased from 7.8 ± 0.6 to 2.2 ± 1.1 (fmoles/10^6 cells) ($p<0.05$) in cells supplemented with 20:5ω3, but increased from 7.8 ± 0.6 to 11.8 ±2.5 (fmoles/10^6 cells) ($p<0.05$) in the cells supplemented with C16:0. Supplementation with 18:2ω6 did not significantly change binding. Scatchard analysis revealed that the inhibition of PAF binding by 20:5ω3 supplementation was mainly due to a decrease in number of PAF receptors (hypothesis 7). Increase in intracellular free calcium concentration in response to PAF in the cells supplemented with 20:5ω3 was also reduced by 60%, suggesting that 20:5ω3 suppresses PAF-mediated signal transduction (hypothesis 8).

To examine the effect of blocking ion conductance on PAF binding to the cells, the epithelial cells were incubated with various ion channel blockers at 37°C for 15 minutes before specific [^{3}H]PAF binding was determined (Chapter VI). Verapamil, a Ca^{++} channel blocker, and amiloride, a Na^+ channel blocker, inhibited specific binding of [^{3}H]PAF to the cells with an IC_{50} of 4-5x10^{-6}M and 2x10^{-4}M respectively. Diphenylamine-2-carboxylate (DPC), a Cl^- channel blocker, dramatically increased PAF binding to the cell in a dose dependent manner. Scatchard analysis revealed that verapamil and amiloride decreased both binding affinity and number

of PAF receptor, while DPC increased PAF binding sites without affecting binding affinity (hypothesis 9).

Modulation of PAF generation by changing membrane fatty acid composition and the effect of eicosanoids on PAF production were examined in a human lung epithelial cell line (A549) (Chapter VII). The human lung epithelial cells were incubated with 100 μM of various fatty acids (16:0, 18:1ω9, 18:2ω6, 20:4ω6 or 20:5ω3) for 6 h to examine the effect of fatty acid supplementation on the agonist-stimulated synthesis of platelet activating factor (PAF). Fatty acid composition of cell membrane was analyzed and level of PAF was determined by radioimmunoassay after stimulation of the cells with a calcium ionophore A23187. Lipid analysis revealed incorporation of each fatty acid into membrane phospholipid. The epithelial cell was found to synthesized PAF upon stimulation with ionophore A23187. The cells supplemented with 20:4ω6 produced significantly higher levels of PAF , while the cells supplemented with 20:5ω3 and 18:1ω9 generated slightly lower levels of PAF. These results suggest that the agonist-stimulated formation of PAF by the cell can be modulated by supplementation of cell with fatty acids (hypothesis 10). Incubation of the cells with various eicosanoids (PGE_2, $PGF_{2\alpha}$, LTB_4, LTC_4 and LTD_4) for 15 min showed that each of them could induce significant formation of PAF, but it appears that PGE_2 and LTB_4 are more effective, indicating an association of eicosanoids with PAF generation (hypothesis 11).

General Discussion

Alpert and Walenga (1991) recently postulated, in light of the low level of 20:4ω6 found in cultured human airway epithelial cells, that these cultured cells may lack Δ6-desaturase. Whether or not human airway epithelial cells have this desaturase activity determines the availability of polyunsaturated fatty acids (e.g. 20:4ω6 and 20:5ω3) for syntheses of phospholipid and lipid mediators. By direct

experiment with labelled precursor fatty acid and lipid analysis, this thesis has clearly demonstrated that human airway epithelial cells are able to desaturate and elongate 18:2ω6 to form 20:4ω6. This thesis has also defined the turnover rate of essential fatty acid in different phospholipid fractions and shown the feasibility of modifying membrane lipid profile by providing exogenous fatty acids. In general, these data indicate active metabolism of essential fatty acid in human airway epithelial cells. This metabolism may play a key role in regulation of membrane properties and function in these cells. It is suggestive that pathologically induced alterations in both membrane fatty acid composition and lipid mediator production in pulmonary disease may, in part, be mediated by changes in activities of incorporation and desaturation of fatty acid and could be normalized by dietary supplementation with specific fatty acid. This thesis has provided basic information of essential fatty acid metabolism in human airway epithelial cells. These data are of importance for understanding abnormalities in both membrane fatty acid composition and lipid mediator generation in pulmonary diseases such as cystic fibrosis and are suggesttive of potential therapy of the disease through dietary fat supplementation.

In cystic fibrosis, both chloride ion impermeability across the epithelial cell membrane and low levels of 18:2ω6 in many tissues and plasma is well known. However, the cause of the low levels of 18:2ω6 in CF is still controversial. Although many studies have suggested that a defect in metabolism of essential fatty acid may be responsible for the abnormality, it is unknown whether impermeability of Cl^- affects incorporation and metabolism of 18:2ω6 in airway epithelium. This thesis demonstrates that making epithelial cell membranes impermeable to Cl^- movement inhibits incorporation of fatty acids into phospholipids of cultured human airway epithelial cells. This novel finding suggests that Cl^-conductance may play a role in fatty acid incorporation into epithelial cell membrane phospholipid and provides insight into the relationship between the low levels of 18:2ω6 in phospholipid and Cl^-

impermeability of the epithelial cell in cystic fibrosis. Based on the finding that blocking the Cl^- channel inhibited 18:2ω6 incorporation into membrane phospholipid it is proposed that low levels of 18:2ω6 observed in phospholipid in CF can be attributed in part to decreased incorporation of the fatty acid resulting from a defect in Cl^-conductance. However. the mechanism by which blocking Cl^- conductance affects fatty acid incorporation into phospholipid remains to be investigated. Future study to examine 18:2ω6 incorporation into cellular lipid in the CF cell is indicated.

Since PAF is a potent inflammatory mediator and has been implicated in the pathogenesis of pulmonary infection, identification of PAF receptor and understanding of regulation of PAF action in human airway epithelial cells are of importance for therapy of pulmonary inflammatory disease. This thesis has provided evidence that human airway epithelial cell have functional PAF receptors. Existence of functional PAF receptors in airway epithelial cells implies that the epithelial cell plays a direct role in the inflammatory process for pulmonary diseases such as asthma and cystic fibrosis. The important findings are that PAF receptor binding can be modulated by supplementation of the cells with different fatty acids and by blocking ion channels. Supplementation of the cells with fatty acid altered fatty acid composition of the membrane phospholipid and the PAF binding site. This suggests that responsiveness of airway epithelial cells to inflammatory stimuli may be regulated by modifying cellular fatty acid composition through dietary fatty acid supplementation. The novel observation is that 20:5ω3 inhibited both PAF binding and PAF-mediated Ca^{++} mobilization, indicating that the anti-inflammatory effects of 20:5ω3 may be contributed to by the suppression of eicosanoid production also by an attenuation of cellular responsiveness due to a reduced ability to transduce signal into an appropriate response. This suggests a novel mechanism by which 20:5ω3 produces an anti-inflammatory effect.

The results that blocking ion channels altered PAF binding in the cells imply

that ion transport across membrane may be involved in the action of PAF at the receptor level, although the mechanism for the interaction between ion conductance and PAF receptor remains to be examined. The new finding that a Cl^--channel blocker increases PAF binding may have relevance to pathological conditions of cystic fibrosis in which both Cl^- impermeability and inflammation occur. It is possible that the defect in Cl^- transport potentiates inflammatory reaction in CF. In this regard, determination of PAF generation in the CF cell is of interest. Furthermore, it is implied that Ca^{++} and Na^+-channel blockers may be beneficial drugs for treatment of PAF-involved inflammatory diseases.

This thesis also demonstrates that agonist-stimulated synthesis of PAF by the human lung epithelial cells can be modulated by altering cellular fatty acid composition. The observation that a number of arachidonate metabolites including LTB_4, LTD_4, LTE_4, PGE_2 and $PGF_{2\alpha}$ were able to stimulate PAF production suggests a close relationship (interaction) between fatty acid, eicosanoids and PAF. These findings, together with the effect of fatty acid on PAF binding, indicate an important role for fatty acids in regulation of inflammatory response by affecting generation and action of inflammatory mediators in lung epithelial cells. For example, 20:5ω3 inhibited PAF binding, PAF-mediated $[Ca^{++}]i$ response and PAF generation, while 20:4ω6 and its metabolites enhanced PAF generation. Long-term comparative studies of the effects of dietary supplementation with fatty acids (e.g. 20:5ω3) on lipid mediator (e.g. LTB_4 and PAF) generation and clinical conditions in a large number of patients with pulmonary inflammatory diseases such as asthma and cystic fibrosis are warranted.

Chapter IX

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APPENDIX

A.1 *Time-dependent changes in labelled fatty acid distribution (refer to Chapter IV)*

To demonstrate a dynamic change in phospholipid fatty acid chains in cultured epithelial cells, distribution of labelled fatty acids (18:2ω6 and its desaturated products) among the major phospholipids (PC, PI and PE) at different time of pulse and chase was analyzed. Figure A.1a shows the alteration in distribution of different labelled fatty acids in individual phospholipids during 8 h pulse incubation with [^{14}C]-18:2ω6. In phosphatidylcholine, the proportion of labelled 18:2ω6 decreased slightly with a slight increase in labelled 18:3ω6 and 20:3ω6, but not 20:4ω6. In both phosphatidylinositol and phosphatidyl-ethanolamine, with longer incubation, a greater proportion of labelled 18:3ω6, 20:3ω6 and 20:4ω6 were found while proportion of labelled 18:2ω6 decreased apparently. These results suggest that desaturated/elongated products of 18:2ω6 accumulate more in PI and PE fractions.

Figure A.1b shows alteration in the amount of labelled 18:2ω6 and its desaturated products in phospholipid fractions during 4 h chase incubation. It is apparent that the amount of unaltered [^{14}C]-18:2ω6 in all phospholipids decreased considerably, while its metabolites changed relatively less in all phospholipid fractions during the chase.

Overall, these results indicated that profile of phospholipid fatty acyl chains changed from time to time as fatty acid was metabolized by the cells.

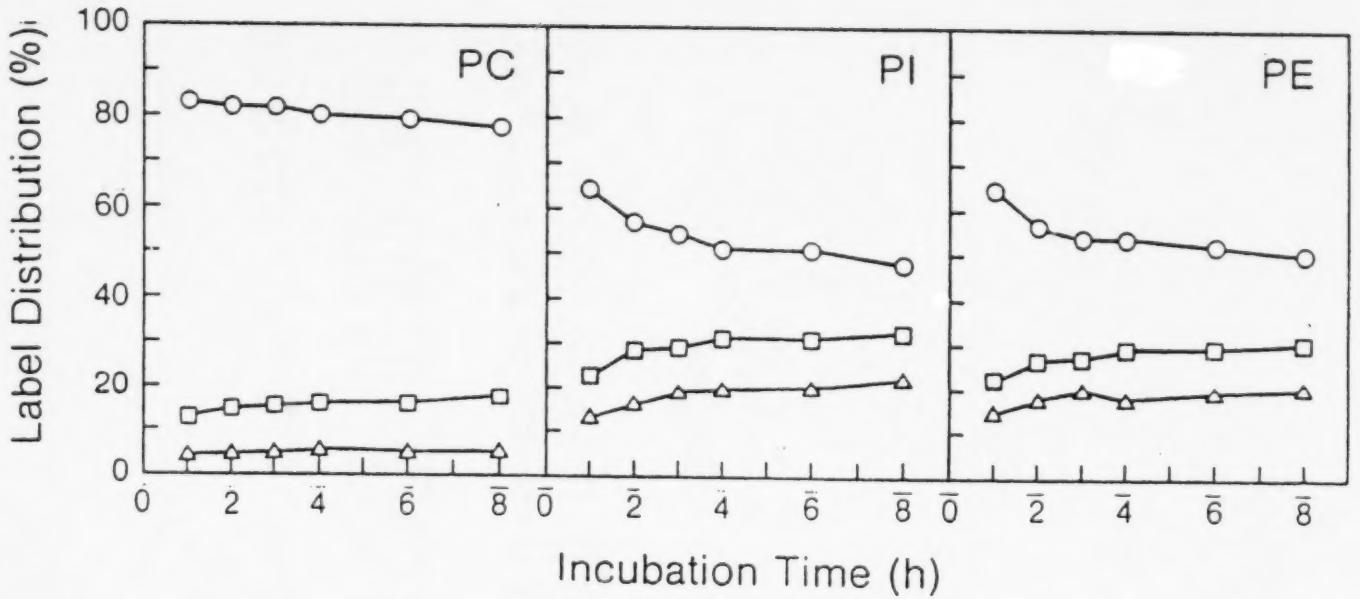

Figure A1a Dynamic alteration in distribution of incorporated [1-^{14}C]18:2ω6 and its products in phospholipids of cultured human airway epithelial cells

Cells were incubated with [1-^{14}C]18:2ω6 (2.2 x 10^6dpm/100 nmol) for various times as indicated. Cells were harvested. Lipids were extracted, seperated on TLC and methylated. Fatty acid methyl esters were seperated on $AgNO_3$-TLC. Labelled fatty acids in silica were scraped and counted by scintillation counter. Values were expressed as percent of radiolabel ini each fatty acid was divided by the sum of the radiolabel in the three fatty acids. Results are the means of two experiments in duplicate. o–o, 18:2ω6; □-□ 18:3ω6 + 20:3ω6 Δ-Δ,20:4ω6 (variation < 10%)

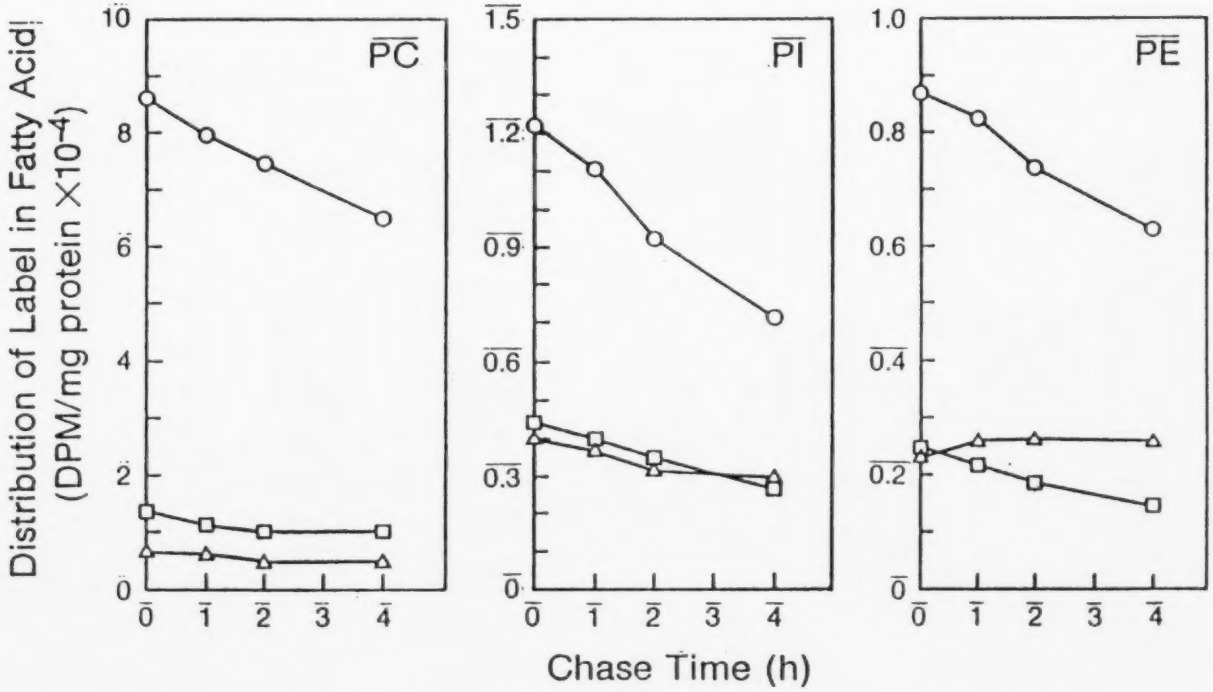

Figure A1b **Alteration in amount of labelled fatty acids of phospholipid fractions during chase incubation**

The experimental conditions were as described for Figure V.1. After various times of chase, cells were harvested. Lipids were extracted, separated on TLC and methylated. Fatty acid methyl-esters of each phospholipid were further separated on $AgNO_3$-TLC plates. Labelled fatty acids in silica were scraped and counted by scintillation counter. Results are the means of two experiments. ○ - ○, 18:2; □ - □, 18:3±20:3; Δ - Δ, 20:4.

A.2 ***Fatty acid composition of individual phospholipids of cultured human airway epithelial cells (refer to Chapter IV)***

The fatty acid composition of each major phospholipid extracted from the cells cultured with medium containing 1% FBS was analyzed by gas chramatography. The pattern of individual phospholipid fatty acid chains is distinct (Table A.2). Overall, in PC there is a higher level of monounsaturated fatty acid (18:1). In PI and PE, there is a higher level of ω6 fatty acids, particularly 20:4ω6. Analysis of the percent of total 18:2ω6 or 20:4ω6 found in phospholipid classes showed that PC, PE, PI and PS contain 58%, 18%, 16% and 8% of total 18:2ω6 and 24%, 43%, 28% and 5% of total 20:4ω6 respectively. It is noted that these primary cultured cells (without fatty acid supplementation) have lower levels of 18:2ω6 and 20:4ω6 in PC fraction when compared with that found in freshly isolated cells (Holtzman,1986).

Table A2

Fatty acid composition of phospholipids from cultured human airway epithelial cells

Fatty acid	Quantity (% w/w) PC	PE	PI	PS
15:0	0.41±0.03	2.7±0.60	0.45±0.2	1.1±0.1
16:0	27.9±4.1	4.9±1.0	6.2±1.4	7.6±1.7
16:1	5.7±1.0	–	1.0±0.2	2.5±0.8
17:0	0.53±0.13	4.0±0.7	5.5±0.2	1.0±0.1
18:0	5.5±0.4	14.6±0.7	25.1±1.7	30.1±2.6
18:1ω9	32.8±4.9	26.6±2.3	14.6±2.8	25.9±2.2
18:1ω7	13.8±2.3	7.4±1.6	7.9±0.9	5.7±1.0
18:2ω6	2.3±0.34	2.6±0.32	3.1±0.5	2.8±0.8
20:2ω6	0.63±0.23	–	2.2±1.5	2.6±0.9
20:3ω9	0.37±0.05	–	2.5±0.7	–
20:3ω6	1.45±0.16	4.9±1.9	6.7±3.5	4.4±1.5
20:4ω6	1.75±0.3	16.4±1.7	14.7±2.8	2.7±0.1
22:5ω6	0.2±0.05	1.4±0.3	1.2±0.6	1.0±0.4
22:5ω3	0.35±0.10	2.8±0.3	1.2±0.17	0.9±0.3
22:6ω3	0.42±0.06	5.1±1.3	1.9±0.31	2.4±0.9
ΣSFA	36.0±3.5	25.5±1.9	36.1±2.0	40.3±2.1
ΣMUFA	55.3±4.6	36.6±3.1	29.6±3.1	40.8±3.4
Σω6	7.2±1.1	29.7±2.6	29.2±4.5	14.6±1.3
Σω3	1.0±0.3	8.2±1.7	5.1±0.5	4.4±0.7

Phospholipids of cultured airway epithelial cells were separated using TLC and then methylated, and the resulting fatty acid methl esters were identified and quantified by gas-liquid chromatography. Values are the means of 4 experiments.

PC=phosphatidylcholine; PE=phosphatidylethanolamine; PI=phosphatidylinositol; PS=phosphatidylserine.

ΣSFA=total saturated fatty acids; ΣMUFA=total monounsaturated fatty acids; Σw6=total w6 fatty acids; Σw3=total w3 fatty acids.

A.3 *Schematic representation of 18:2ω6 metabolism in human airway epithelial cells (refer to Chapter VI)*

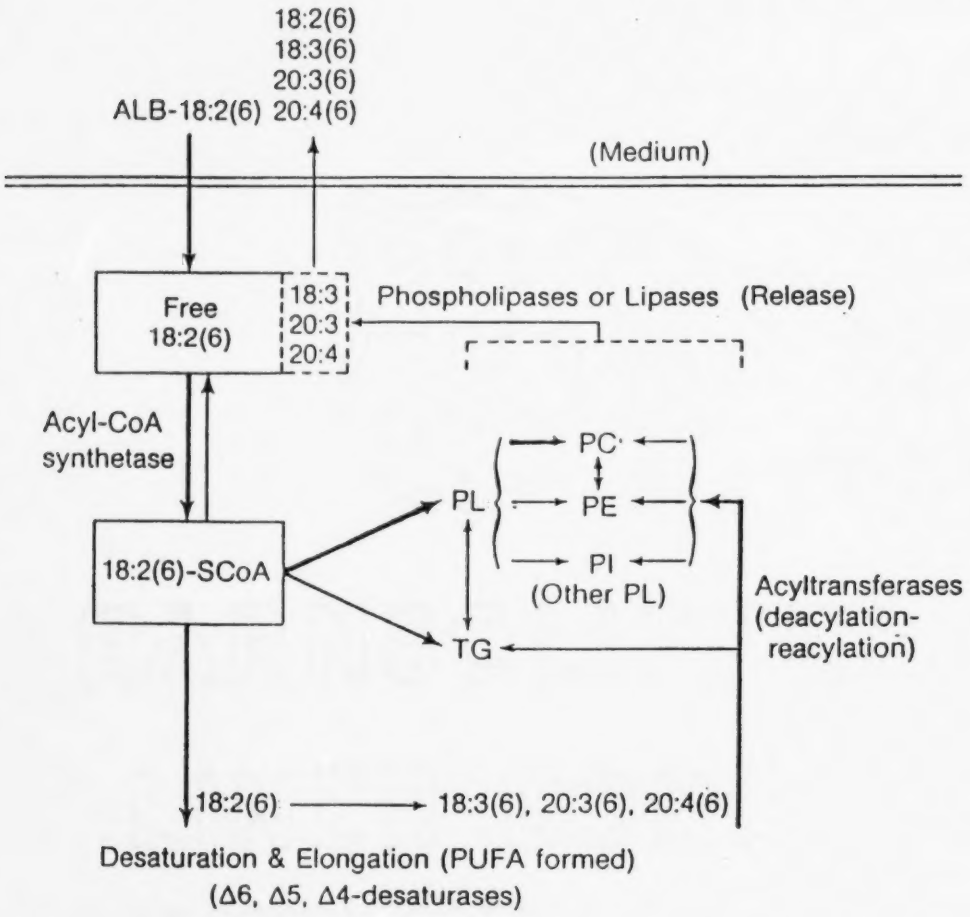

Figure A.3 Schematic representation of 18:2ω6 metabolism in human epithelial cells.

Extracellular free 18:2ω6 is taken up by cells. Inside the cell, 18:2ω6 is converted, by acyl-CoA synthetase, to 18:2ω6-sCoA, which can either incorporates into phospholipid (mostly in PC) and triglyceride, or be catalyzed by desaturases to form higher unsaturated fatty acids (20:3ω6, 20:4ω6). The desaturated products further incorporate into phospholipids, particularly PE and PI, and triglyceride by acytransferases, The incorporated fatty acids can be released by phospholipases or lipases. A number of interactions or exchanges between different fatty acid pool occur in above metabolic processes.